STRUCTURAL ELUCIDATION OF POLYAMINE CONTAINING NATURAL PRODUCTS BY HPLC-MS

Dissertation

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Promotionskomitee: Prof. Dr. Heinz Heimgartner (Vorsitz), Prof. Dr. Stefan Bienz (Leitung der Dissertation), Dr. Laurent Bigler

Ο ανιξερος διαβεβαιωνει, ο επιστημονας αμφιβαλλει, ο σοφος σκεπτεται.

Αριστοτελης (384–322 π.Χ.)

L'ignorant affirme, le savant doute, le sage réfléchit.

Aristote (384–322 av. J.-C.)

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PREFACE

This thesis describes the MS/MS investigation of polyamine spider toxins and related compounds. It consists of an introductory paragraph, which deals with the collection of spider venom, and of four separate chapters describing our detailed analytical investigations. Each chapter is independent from the others and has its own numbering of the schemes, figures, substances, and references.

Chapter 1 describes the synthesis of a small library of naturally occurring acylpentamines and the correlation of their HPLC-MS and -MS/MS behavior with components found in the venom of the spider *Agelenopsis aperta*. This chapter is published in *Helv. Chim. Acta* **2002**, *85*, 2827. *Chapter 2* discusses the MS/MS fragmentation mechanisms of acylpentamines and their ^{15}N-labeled derivatives to explain the origin of several unexpected fragment ions. This chapter is published in *J. Am. Soc. Mass Spectrom*. **2004**, *15*, 1636. *Chapter 3* presents the detection of linear free polyamines and their N-hydroxylated, guanidylated, and acetylated derivatives by HPLC-MS in the venom of the spiders *Agelenopsis aperta*, *Hololena curta*, and *Paracoelotes birulai*. This chapter is published in *Toxicon* **2005**, *46*, 0000. *Chapter 4*, finally describes the isolation, structure elucidation, MS/MS fragmentation behavior, and synthesis of the two new spermidine alkaloids, Chisitine 1 and 2. This chapter is published in *Helv. Chim. Acta* **2004**, *87*, 1411.

The synthetic parts to the several acylpolyamines used in *Chapter 1* and *2* of this thesis were contributed by Dr. N. Manov and are also parts of his Ph.D. thesis (2003). They are included in this manuscript as a part of the published papers and to place our MS/MS investigations into a proper context.

SUMMARY

Polyamine-containing natural products are found in many animals and plants. For instance, spider venoms — whereon we focused our research — are known to be a rich source of acylpolyamine toxins. These biologically active compounds interact, *e.g.*, with ion channels in the mammalian central nervous system and are thus of therapeutical interest for the study of brain disorders such as *Parkinson*'s and *Alzheimer*'s diseases. The toxins are found in the spider venoms within complex mixture of several types of compounds and they are available in trace amounts only. Therefore, sophisticated and highly sensitive analytical methods are required to detect and analyze these compounds. In the recent years, high-performance liquid chromatography coupled with mass spectrometry (HPLC-MS) has found a wide application in the detection of low abundant natural products, and particularly in the investigation of acylpolyamines in spider venoms. In combination with tandem-mass spectrometry (MS/MS or MS^n), efficient structural elucidation of the analytes was achieved.

To rely the structural elucidation of polyamine toxins on MS-methods alone, however, the fragmentation behavior of the sample molecules has to be well understood. To learn more about their behavior, the MS/MS data of structurally well defined and chemically pure reference compounds have to be collected and studied. For this purpose, synthesis of the toxins and model compounds is often necessary.

This thesis presents the structural elucidation of several polyamine containing natural products found in spider venoms and in the leaves of a tree, based essentially on HPLC-MS and -MS/MS experiments.

Chapter 1 discusses the results of our investigation of the venom of the spider *Agelenopsis aperta*. We were able to detect some acylpentamine toxins postulated to be contained in the venom in addition to those previously reported. To do so, we used a library of twelve synthetically pure reference compounds prepared by solid-phase synthesis. The library consisted of four linear pentamine isomers, each acylated with three different head acyl groups. These synthetic compounds were compared with respect to their chromatographic and MS/MS behavior with the components of the natural toxin mixture. To investigate the chromatographic behavior, the synthetic references were admixed to the natural venom, and the increase of the chromatographic peaks was monitored (*Figure 1*). With such comparative studies, we

confirmed the presence and structure assignments of seven known toxins, and we were able to detect five new pentamine derivatives within the complex venom mixture. The study of the MS/MS data of the several pure synthetic isomers revealed a characteristic fragmentation pattern for the acylpentamines. The typical MS/MS for each isomer is a particular consequence of the location of the 'tetramethylene unit' within the polyamine backbone, since the 1,4-diaminobutane moiety dominates the fragmentation behavior of the compounds. Our findings allowed the characterization of new polyamine derivatives without the need for their chemical synthesis.

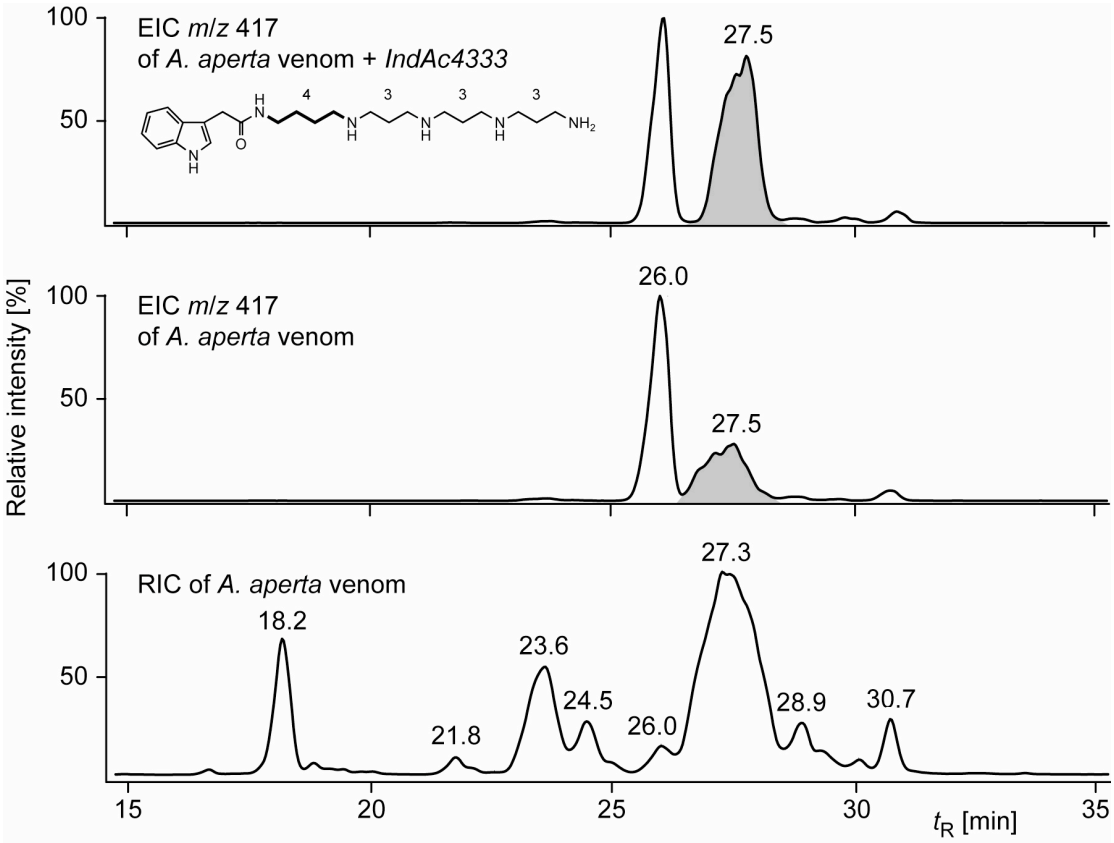


Figure 1. *Extracted ion chromatograms (EIC) of the ions at* m/z *417 in natural venom of* A. aperta *and— exemplarily — in spiked venom with synthetic* IndAc4333*.*

Chapter 2 discusses the origin of a series of unexpected fragment ions that were found in the MS/MS of the acylpentamines described in *Chapter 1*. The identity of these ions is crucial since they could lead to misinterpretation of the fragmentation spectra and consequently to incorrect structure assignments of the toxins. For this study, three ^{15}N-labeled isomers of *IndAc3334* were prepared by solid-phase synthesis and investigated by MS/MS. The three isomers were carrying the labeled N-atom at three different positions within the polyamine backbone. The MS/MS of the labeled compounds allowed, *e.g.*, to identify the internal portion of the molecule highlighted in *IndAc33*^{15}*N34* of *Figure 2* to be contained in the unexpected signal ^{15}N-$\mathbf{b_{33}}$.

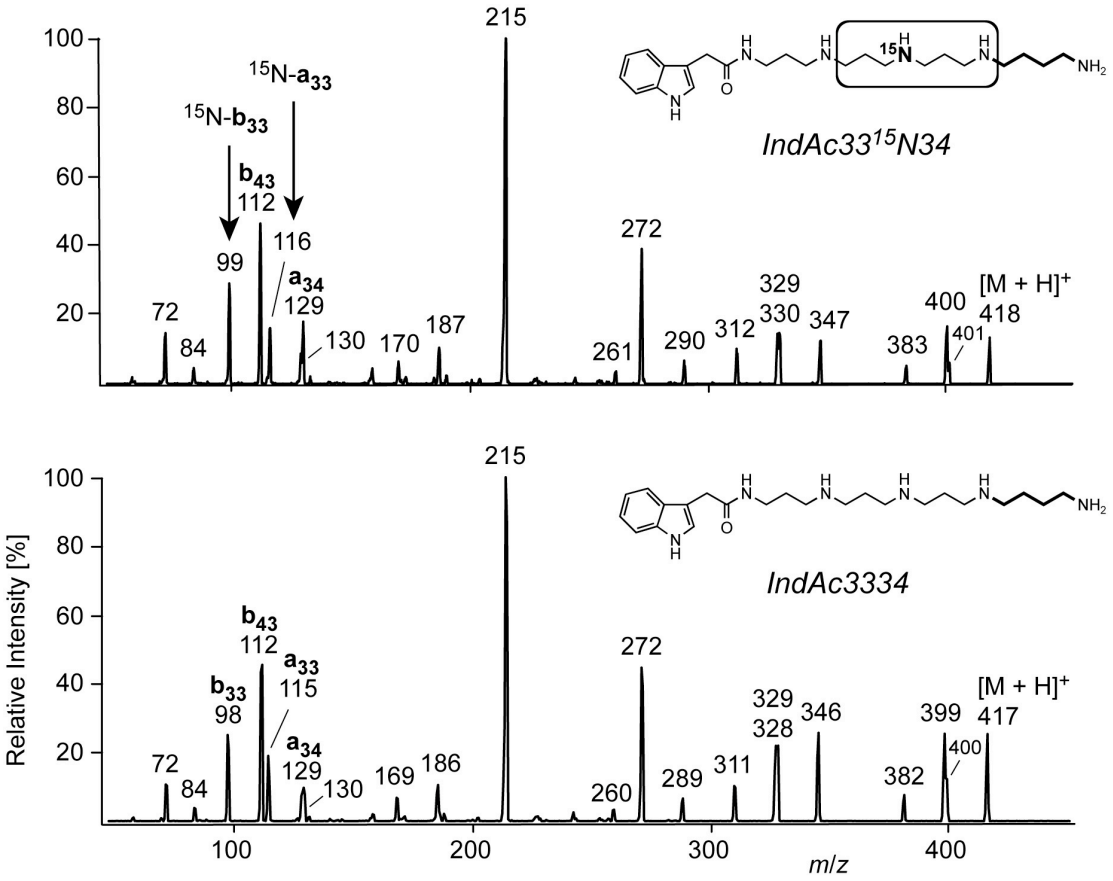
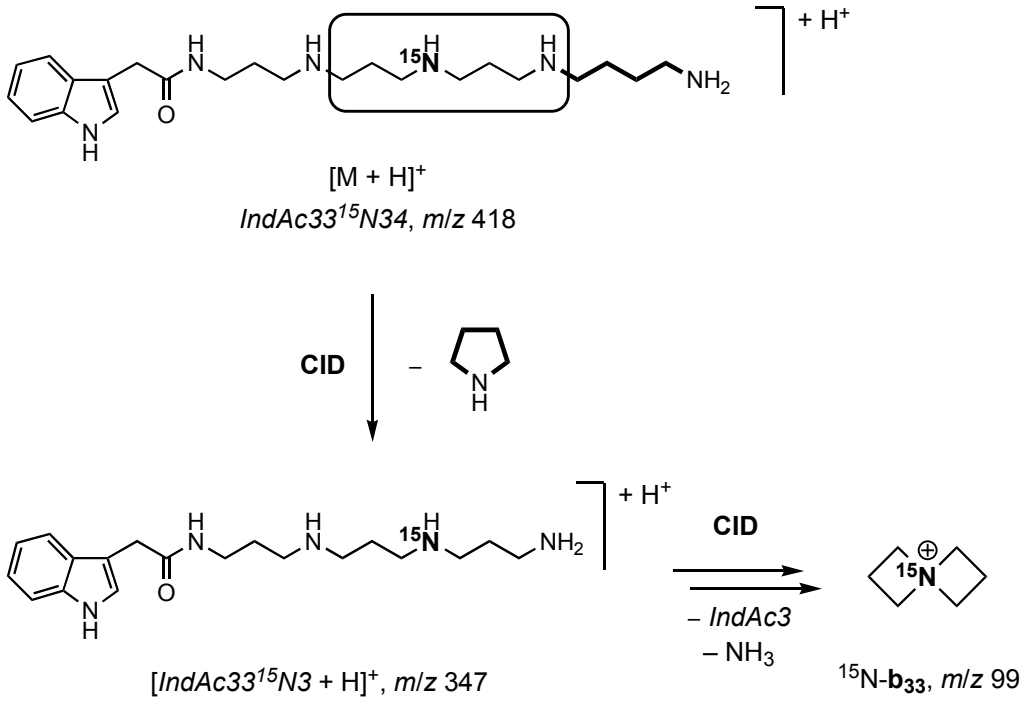


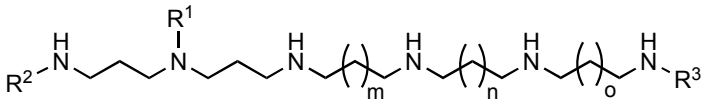
Figure 2. *MS/MS spectra of the pentamine derivative* IndAc3334 *and its* ^{15}*N-labeled analog* IndAc33^{15}N34.



Scheme 1. *Sequential fragmentation mechanism of the pentamine derivative* IndAc33^{15}N34 *during MS/MS.*

From this observation, a sequential fragmentation mechanism (*Scheme 1*) as well as a rearrangement/fragmentation mechanism were proposed for the decomposition of acylpolyamines during MS/MS.

Chapter 3 presents the investigation of early HPLC-fractions of the venom of the spiders *Agelenopsis aperta*, *Hololena curta* and *Paracoelotes birulai* containing linear polyamines. This study was motivated by the assumption that linear polyamines are the biosynthetic precursors of the parent acylpolyamines. Due to the absence of UV-activity, the detection of the non-acylated polyamine compounds had to be based solely on HPLC-MS and -MS/MS. As a result, we detected in addition to underivatized compounds, thirteen new N-hydroxylated, guanidylated or acetylated polyamines (*Figure 3*). Furthermore, *Chapter 3* discusses a possible biosynthesis of acylpolyamines in spider venoms.

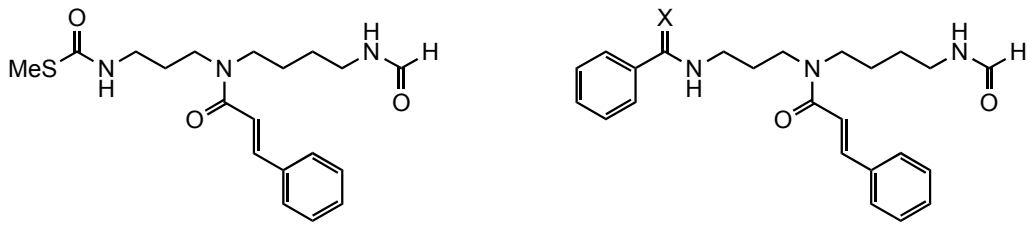


R^1 = H; OH R^2 = H; Ac R^3 = H; Gu; Ac

m,n,o = 2,1,1; 1,2,1; or 1,1,2

Figure 3. *Structures of the linear hexamine derivatives detected in* A. aperta, H. curta, *and* P. birulai.

In *Chapter 4*, we describe the structural elucidation of two new trisubstituted spermidine alkaloids, isolated from a methanolic extract of the leaves of the southern Irian Jaya tree *Chisocheton weinlandii*. The two compounds, named Chisitine 1 and Chisitine 2 (*Figure 4*), were characterized by NMR spectroscopy, HPLC-MS, and HPLC-MS^n.



Chisitine 1

X = O : Chisitine 2

X = ^{18}O : [^{18}O]-Chistine 2

Figure 4. *Structure of the spermidine alkaloids Chisitine 1 and 2 isolated from* C. weinlandii, *and of a synthetic* ^{18}*O-labeled Chisitine 2 isotopomer.*

Moreover, the structure elucidation of the chisitines was secured by synthesis of the compounds and by their comparison with the natural products. *Chapter 5* discusses the MS^n fragmentation scheme of Chisitine 2 based on H/D-exchange experiments and the investigation of an ^{18}O-labeled analog. The study will possibly allow the detection of additional related polyamine alkaloids from plant material, due to their characteristic MS/MS fragmentation behavior.

ZUSAMMENFASSUNG

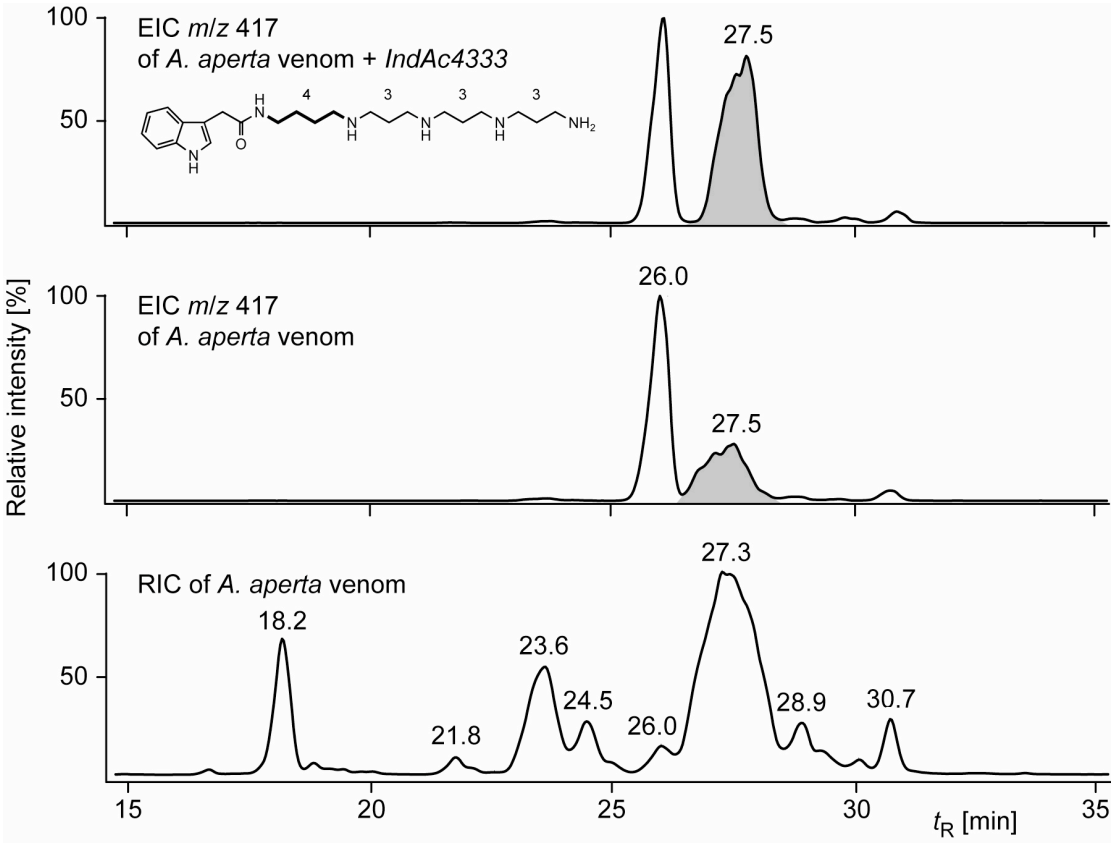
Naturprodukte, die Polyamine enthalten, werden bei zahlreichen Tier- und Pflanzenarten gefunden. Zum Beispiel Spinnengifte — auf welche wir unsere Forschungen fokussierten — sind bekannt als reichhaltige Quellen von Acylpolyamin-Toxinen. Diese biologisch aktiven Substanzen beeinflussen z.B. Ionen-Kanäle im zentralen Nervensystem von Säugetieren und sind deshalb wertvoll für potenzielle therapeutische Zwecke im Zusammenhang mit Gehirnerkrankungen wie der *Parkinson*- oder *Alzheimer*-Krankheit. Spinnengifte stellen eine komplexe Mischung aus verschiedenen Typen von Verbindungen dar, wobei Polyamin-Toxine nur einen kleinen Teil davon ausmachen. Aus diesem Grund sind hoch entwickelte und empfindliche analytische Methoden nötig, um diese nur in kleinsten Mengen vorhandenen Komponenten zu detektieren und zu analysieren. In den letzten Jahren hat dazu die Hochleistungs-Flüssigkeitschromatographie gekoppelt mit Massenspektrometrie (HPLC-MS) eine spezielle Bedeutung erhalten. Neben der reinen Detektion von in spuren vorliegenden Substanzen erlaubt die Methode, in Kombination mit der Tandem-Massenspektrometrie (MS/MS oder MS^n) zusätzlich die Strukturaufklärung der Probemoleküle.

Damit die Struktur von Polyamin-Toxinen alleine auf der Basis von MS-Methoden bestimmt werden kann, muss das Fragmentierungsverhalten der untersuchten Verbindungen gut verstanden sein. Um dieses Fragmentierungsverhalten verstehen zu lernen, müssen die MS/MS-Daten der Naturprodukte analysiert und mit Messdaten von bekannten und chemisch reinen Referenzverbindungen verglichen werden. Zu diesem Zweck müssen oft spezifische Toxine oder Modellmoleküle synthetisiert werden.

Diese Dissertation zeigt die Strukturaufklärung von verschiedenen Polyamin-Naturprodukten auf, die in Spinnengiften oder in den Blättern eines Baumes vorkommen. Die Untersuchungen stützen sich im wesentliche auf HPLC-MS und -MS/MS Experimente.

In *Kapitel 1* wird der Nachweis von Acylpolyaminen im Spinnengift von *Agelenopsis aperta* aufgezeigt. Acylpolyamine wurden bereits früher im Gift von *A. aperta* gefunden, einige davon konnten jedoch strukturell nicht eindeutig nachgewiesen werden. Wir benötigten für unseren Nachweis zwölf synthetisch hergestellte, reine

Verbindungen, die mit Festphasensynthese erhalten wurden. Die chromatographischen und MS/MS-Eigenschaften der synthetischen Komponenten wurden mit denjenigen der natürlichen Komponenten des Spinnengiftes verglichen. Um das chromatographische Verhalten zu untersuchen, wurden die synthetischen Verbindungen mit dem Spinnengift gemischt (*Figur 1*). Unsere Sammlung von Polyaminderivate beinhaltete jeweils vier lineare Acylpentamin-Isomere, welche mit je drei verschiedenen Acyl-Gruppen gekoppelt waren. Anhand der Vergleichsmethode, die wir bei dieser Studie herangezogen haben, konnten wir die Existenz der bereits bekannten sieben Verbindungen nachweisen und zusätzlich fünf neue Acylpentaminderivate im komplexen Spinnengift bestimmen.

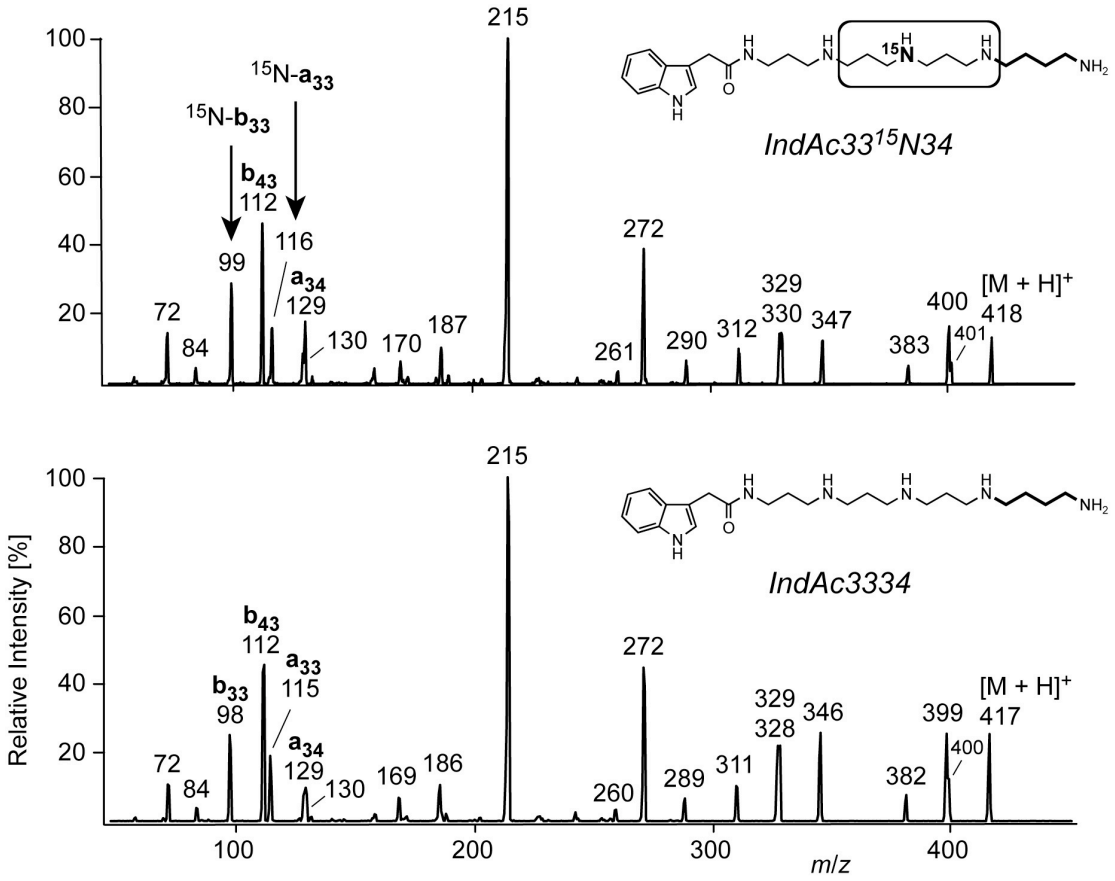


Figur 1. *"Extracted ion chromatograms" (EIC) der Ionen* m/z *417 in natürlichem und in mit synthetischem* IndAc4333 *gemischtem Gift von* A. aperta.

Die Untersuchung der verschiedenen synthetischen Isomere mittels MS/MS hat gezeigt, dass die einzelnen Verbindungen ganz unterschiedliche, charakteristische Fragmentierungsmuster zeigen. Die Charakteristik der MS/MS-Spektren der einzelnen Isomere ist jeweils eine Konsequenz der Position der "Tetramethylen-Einheit" in der Polyaminkette, weil diese 1,4-Diaminobutan-Einheit das Fragmentierungsverhalten der Polyamine dominiert. Die Ergebnisse dieser

Untersuchung erlauben es uns nun neue Polyaminderivate zu charakterisieren, ohne sie synthetisieren zu müssen.

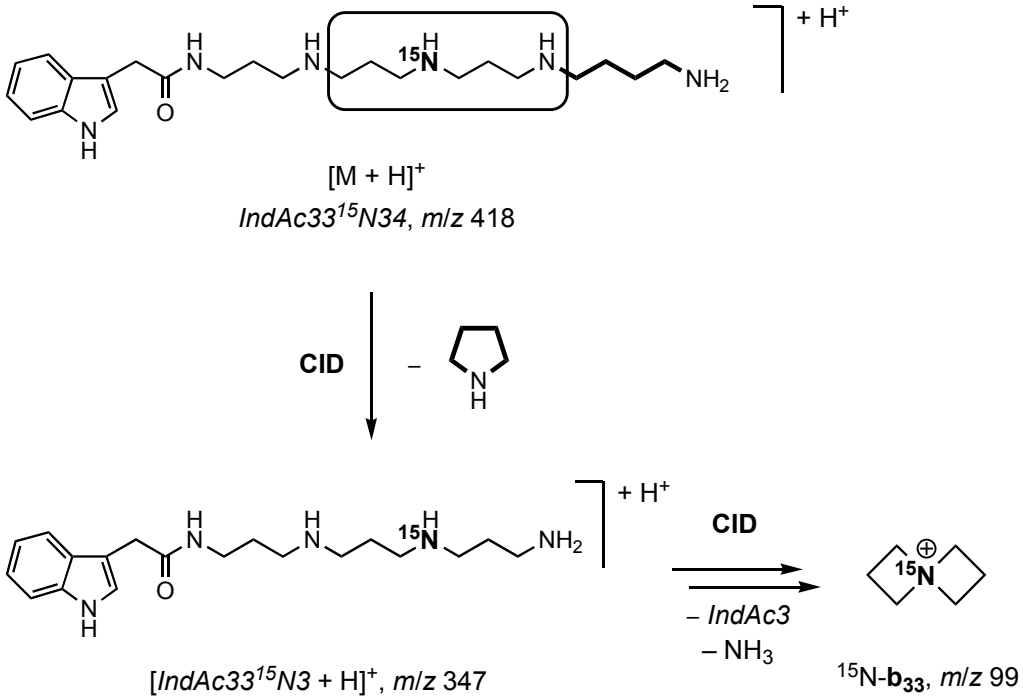
Kapitel 2 berichtet über die Herkunft unerwarteter Fragment-Ionen, die in den MS/MS-Spektren der synthetischen Acylpentaminen (*Kapitel 1*) gefunden wurden. Die Identifizierung dieser Ionen ist wichtig, da es in Unkenntnis ihrer Entstehungsweise zu Fehlinterpretationen bei der Analyse von Fragmentierungs-Spektren und somit zu falschen Strukturzuordnungen bei der Untersuchung von Polamin-Toxine führen könnte. In dieser Studie wurden drei ^{15}N-Isotopen-markierte Isomere von *IndAc3334* mittels Festphasensynthese hergestellt und mit Tandem-Massenspektrometrie untersucht.



Figur 2. *MS/MS-Spektren von Verbindung* IndAc3334 *und von* ^{15}*N-Isotopenmarkierter* IndAc33^{15}N34.

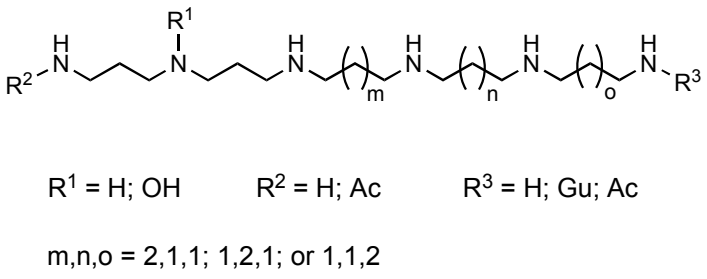
Das ^{15}N-Isotop wurde an drei verschiedenen Stellen der Polyaminkette eingesetzt. Die Interpretation der MS/MS-Spektren dieser Substanzen ermöglichten es uns zu zeigen, dass ein vorher schwierig zu erklärendes Fragment-Ion aus dem internen Teil der Polyaminkette von *IndAc33^{15}N34* stammt (siehe *Figur 2*). Aus diesen Beobachtungen wurden ein sequenzieller Fragmentierungs-Mechanismus (*Schema 1*)

und ein Umlagerungs/Fragmentierungs-Mechanismus vorgeschlagen, welche die Abspaltungen von Acylpolyaminen in MS/MS Experimenten erklären können.



Schema 1. *Sequenzieller MS/MS Fragmentierungs-Mechanismus für das Pentaminderivat* IndAc33^{15}N34.

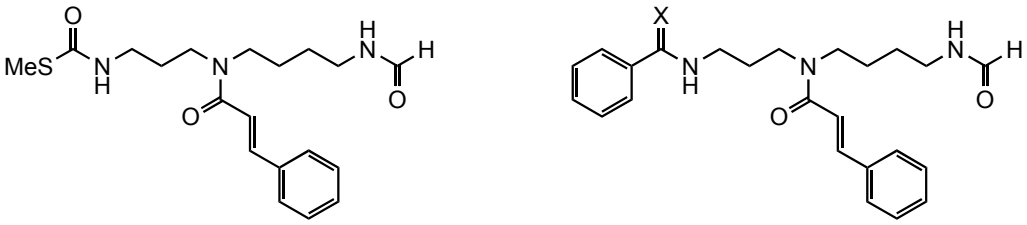
In *Kapitel 3* wird die Detektion lineare Polyamine im Spinnengift von *Agelenopsis aperta*, *Hololena curta* and *Paracoelotes birulai* aufgezeigt. Diese Studie wurde aufgrund der Vermutung durchgeführt, dass lineare Polyamine die biosynthetischen Vorläufer der entsprechenden Acylpolyamine sein könnten. Da die freien Polyamine keinen Chromophor besitzen, ist der Nachweis solchen Komponenten auf reine HPLC-MS- und -MS/MS-Daten zu beziehen. Neben unacylierten Verbindungen konnten dreizehn neue N-hydroxylierte, guanidylierte oder acetylierte Polyamine gefunden werden (*Figur 3*).



Figur 3. *Strukturen von linearen Hexaminderivaten, welche im Spinnengift von* A. aperta, H. curta*, und* P. birulai *gefunden wurden.*

Des Weiteren berichtet *Kapitel 3* über unsere Vorstellungen zum Biosynthese von Acylpolyaminen in Spinnengiften.

In *Kapitel 4* wird die Strukturaufklärung von zwei neuen tri-substituierten Spermidin-Alkaloiden beschrieben, die in einem Blätterextrakt des südlichen Irian Jaya Baumes *Chisocheton weinlandi* gefunden wurden. Die zwei Verbindungen, genannt Chisitin 1 und Chisitin 2 (*Figur 4*), wurden mit NMR-Spektroskopie, HPLC-MS, und -MS^n charakterisiert. Zudem wurden beide Verbindungen synthetisiert und die analytischen Daten der synthetischen Proben mit denjenigen der Naturprodukte verglichen. *Kapitel 4* berichtet zudem über den Fragmentierungsweg von Chisitin 2 im MS/MS, welcher auf der Basis von H/D-Austausch-Experimenten und der Untersuchung eines ^{18}O-markierten synthetischen Analogons abgeleitet wurde. Die Resultate können für die Strukturaufklärung von weiteren ähnlichen Polyamin-Alkaloiden aus Pflanzen herangezogen werden.



Chisitin 1

X = O : Chisitin 2
X = ^{18}O : [^{18}O]-Chistin 2

Figur 4. *Struktur der Spermidin-Alkaloide Chisitin 1 und 2 aus* C. weinlandii *und des synthetisch hergestellten ^{18}O-markierten Chisitins 2.*

COLLECTION OF SPIDER VENOMS

Spiders are an incredibly diverse yet largely unexplored source for the discovery of novel and useful venom toxins with unique properties. To promote their exploration, several farms got specialized in breeding of spiders— as well as of other arthropods such as scorpions —. The venoms used for our investigations were obtained from *Fauna Laboratories, Ltd.*[1], in Almaty (Kazakhstan). In general, juvenile spiders are grown up at very high densities in plastic cups during one or two instars. Once adult, the spiders are separated and individually housed in plastic cups where they develop well if they obtain sufficient prey and are kept under suitable external conditions. The spiders in question — *Agelenopsis aperta, Hololena curta,* and *Paracoelotes birulai* — are held optimally at 27° and 60% relative humidity, they are fed with housefly maggots or wax worm larvae and crickets.

Among the several methods reported to collect venom from spiders, electro-stimulated "milking" is the preferred one. There are several reasons for this: (1) it is assumed that milked venoms, as compared to venoms obtained by gland dissection and extraction, are more similar to the actual venoms spiders use to paralyze or kill their prey; (2) the venoms should be less contaminated by extraneous material like residues of cell debris and digestive fluids, containing proteases and enzymes that may lead to degradation of the toxins present in the venoms; and (3) the spiders have not to be killed and can repetitively be used for milking in periods ranging in general from one to three weeks.

To obtain the venoms for our investigations, the respective spiders (*Agelenopsis aperta*, *Hololena curta*, and *Paracoelotes birulai*) were first anesthetized by exposure to chloroform or carbon dioxide for a couple of minutes. Once motionless, the spiders were brought with modified tweezers under a dissecting microscope with typically 20 times magnification. The first prong of the tweezers, covered with a saline-soaked foam pad, was placed against the sternum, the second insulated one against the carapace just behind the eyes of the arachnidae. By holding the spiders upside down (ventrum facing the operator), the chelicerae and fangs were rinsed with a jet of water using a syringe to remove any residual digestive fluids (*Figure 1*). The

[1] Adress: Dr. Andrey Fedorov, Fauna Laboratories, Ltd., Academgorodok, Institute of Zoology, 480060 Almaty, Kazakhstan. Internet homepage: http://www.faunalabs.com.

chelicerae and fangs were then meticulously examined for debris or moisture and, if needed, rinsed with water again.

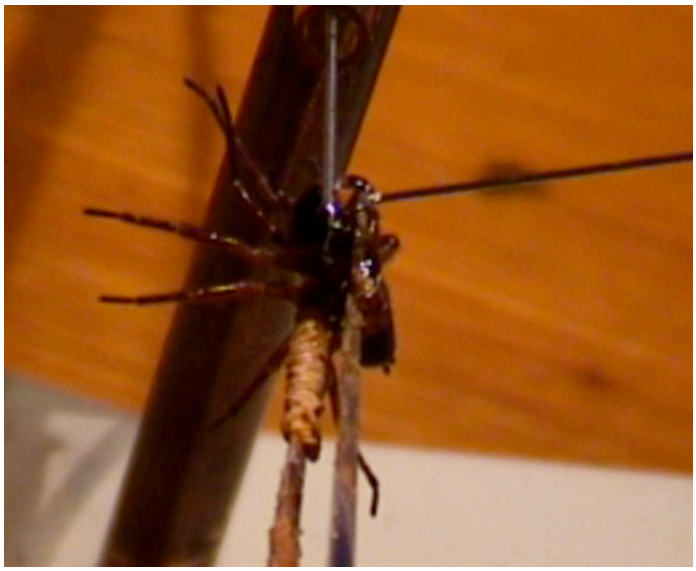


Figure 1. *Rinsing of the chelicerae and fangs of the spider* Paracoelotes birulai *to remove any residual digestive fluid.*

After this cleaning procedure, the chelicerae of the spiders were leant against an electrically conducting pin and "rejection" of the venom is stimulated by applying successive low current electrical shocks. In most cases, a simple foot-switch controlled stimulator, delivering a 60 Hz alternating current of 50–150 mA was used. The expelled venom was directly collected from the fangs in a capillary glass tube (*Figure* 2).

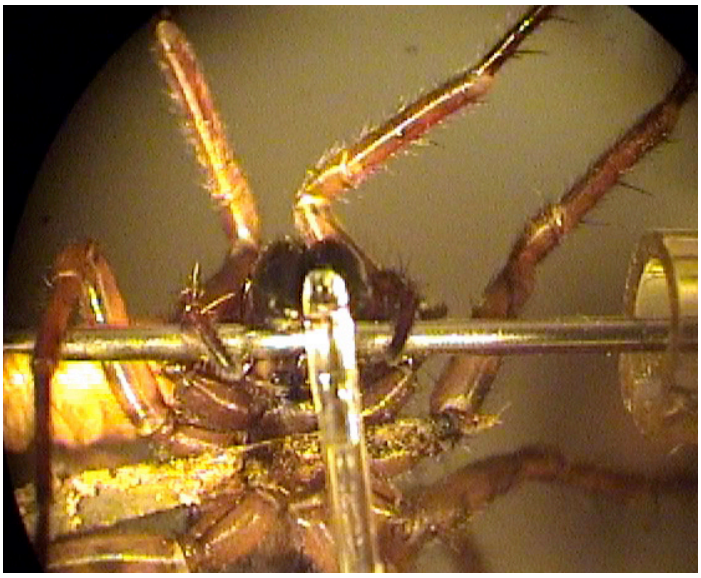


Figure 2. *Microscope view of the venom collected from the spider* Paracoelotes birulai.

The process of milking was observed directly under the microscope to ensure that the capillary comes in contact solely with the chelicerae and fangs. Typically, the investigated spiders yielded approximately from 0.2 to 0.5 μl of venom per milking. After the milking, the spiders were placed back into their habitats and the animals recovered within a few minutes. In a milking session, the milking operation was usually performed successively with eight to sixteen individuals of the same species at intervals of about 20 seconds to one minute, collecting the venom in a single capillary tube. The combined venom (2–10 μl) was then aspirated from the glass capillary into a vial held at –20°. The fluid was lyophilized and produced a colorless powder (1–3 mg) which was stored at –80° to avoid decomposition. To obtain larger amounts of venom, the spider colonies were repetitively milked in periods ranging in general from one to three weeks.

The amount of venoms to be investigated at our disposal are summarized in the following table:

Spider	Family	Volume [μl]	Amount [mg]
Agelenopsis aperta	Agelenidae	500	100
Hololena curta	Agelenidae	50	10
Paracoelotes birulai	Amaurobiidae	50	10

CHAPTER 1

Solid-Phase Synthesis of Polyamine Spider Toxins from the Center and Correlation with the Natural Products by HPLC-MS/MS[1]

Abstract

A recently developed new and divergent approach for the solid-phase synthesis of polyamines and polyamine derivatives was extended to the preparation of linear pentamines, and it was applied to the synthesis of three quartets of isomeric polyamine spider toxins. The twelve synthetic acylpolyamines were investigated by HPLC-UV(DAD)-MS and HPLC-UV(DAD)-MS/MS and compared with the natural products in the complex mixture of the venom of *Agelenopsis aperta*. The comparative investigation supported the structures and assignments of seven previously found toxins and allowed the identification of an additional five polyamine derivatives in the natural sample. The MS/MS study of the isomerically pure polyamine derivatives revealed furthermore a characteristic pattern for the fragmentation of these compounds, which can possibly be used as an evidence in the trace-analysis of other polyamine derivatives.

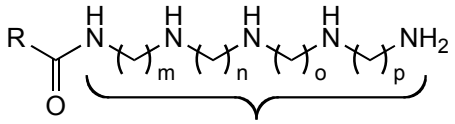
Introduction

Linear polyamines like putrescine, spermidine, and spermine, but also more uncommon representatives, are widely distributed throughout the animal and plant kingdoms. They occur either free in protonated form or as conjugates connected to other biomolecules. Since they exhibit in either form a variety of biological activities [1-4], it is not surprising that polyamines as well as their analogs and derivatives are considered as therapeutic leads for the treatment of a variety of diseases. Unfortunately, natural polyamine derivatives arise often in small amounts and frequently within complex mixtures only. This makes their use in systematic biological investigations unviable. To study the detailed function and action of polyamine compounds, it is, thus, of particular interest not only to have access to a broad spectrum of different polyamine derivatives, preferably also to new substrates

[1] N. Manov, M. Tzouros, S. Chesnov, L. Bigler, S. Bienz, *Helv. Chim. Acta* **2002**, *85*, 2827.

from natural sources, but also to sufficient amounts of pure samples. Highly sophisticated analytical methods to find new lead structures in nature combined with synthetic protocols to provide the respective compounds in ample amounts are, therefore, the optimal team players to support biological investigations related to the polyamines.

Recently, we have been increasingly engaged in the analysis and characterization of polyamine toxins from spiders, in particular from *Agelenopsis aperta* [5] and from *Paracoelotes birulai* [6], by on-line coupled high-performance liquid chromatography and atmospheric-pressure chemical ionization mass spectrometry (HPLC-UV(DAD)-APCI-MS and MS/MS). In the venom of *A. aperta, e.g.*, as many as 33 acylpolyamines with 11 different molecular masses were detected by this method. Most of the structures were securely elucidated by MS/MS, but some evaded explicit determination.



		trivial name	name of nat. product
Type **A**	*PA3334*	*IndAc3334*	(**AG 416a**)
		4-OH-Bz3334	(**AG 379**)
		2,5-(OH)$_2$-Bz3334	(**AG 395b**)
Type **B**	*PA3343*	*IndAc3343*	(**AG 416**)
		4-OH-Bz3343	(—)
		2,5-(OH)$_2$-Bz3343	(—)
Type **C**	*PA3433*	*IndAc3433*	(—)
		4-OH-Bz3433	(—)
		2,5-(OH)$_2$-Bz3433	(—)
Type **D**	*PA4333*	*IndAc4333*	(**AG 416b**)
		4-OH-Bz4333	(**AG 379a**)
		2,5-(OH)$_2$-Bz4333	(**AG 395c**)

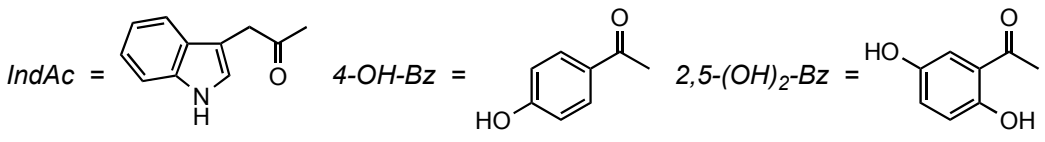


Figure 1. *Typification, structures, and names of the acylpentamines found partially in the venom of the spider* A. aperta.

It was found that the MS/MS analysis of several venom 'components' (selected by their retention times in HPLC and their ion masses) do not conform completely to the fragmentation patterns that would be expected for 'pure sample ions'. Such irregularities have been interpreted as the manifestation of overlapping spectra

deriving from co-eluting compounds contained in the natural toxin mixture. For *IndAc* pentamines it was, *e.g.*, cautiously concluded that *IndAc3334* (**AG 416a**, compound of type **A**) and *IndAc3343* (**AG 416**, compound of type **B**) are such co-eluting components of the venom (*Figure 1*). The isomeric derivative *IndAc4333* (**AG 416b**, compound of type **D**), with a different chromatographic behavior, was also uncovered in the venom, while the remaining isomer *IndAc3433* (compound of type **C**), was not identified. While the structural elucidations for *IndAc3334* and *IndAc4333* appear quite conclusive, the evidence for the constitution of *IndAc3343* (**AG 416**) is rather weak. The assignment is based more on literature precedence — the compound was considered one of the major constituents of the venom of *A. aperta* [7] and of *H. curta* [8] — than on spectroscopic evidence. As a matter of fact, the MS/MS response at *m*/*z* 343, taken as the diagnostic signal for a fragment deriving from *IndAc3343*, could likewise be attributed to a fragment of equal mass deriving from *IndAc3433* (*Figure 2*). Additionally, the signal at *m*/*z* 115, which was regarded as characteristic for the *PA33* terminus in *IndAc4333* (**AG 416b**), would indicate the presence of a structure also possessing a terminal *PA33* unit. Such a compound would be *IndAc3433* rather than *IndAc3343*.

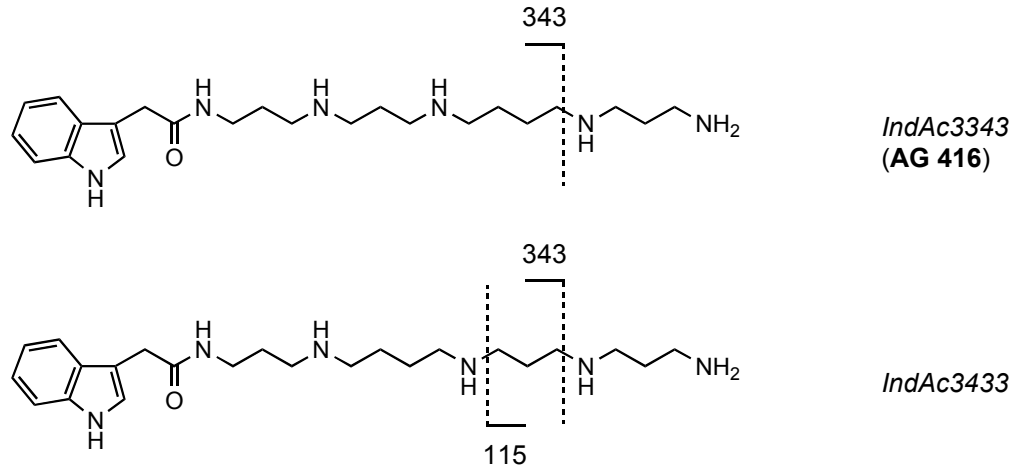


Figure 2. *Proposed indicative fragment of* IndAc3343 (***AG 416***) *and fragments potentially derived from* IndAc3433.

Since we have only marginal knowledge of the fragmentation behavior of the acylpolyamines, it cannot be excluded that the 'unusual' signals in the MS/MS spectra are not due to co-eluting isomeric compounds. They could have also been generated by unexpected fragmentation reactions occurring with the structurally secured *IndAc3334*. To unambiguously establish (or disprove) the presence and structures of the proposed indole-acetamides within the natural venom — eventually to find the remaining isomer *IndAc3433*, too — the respective compounds were

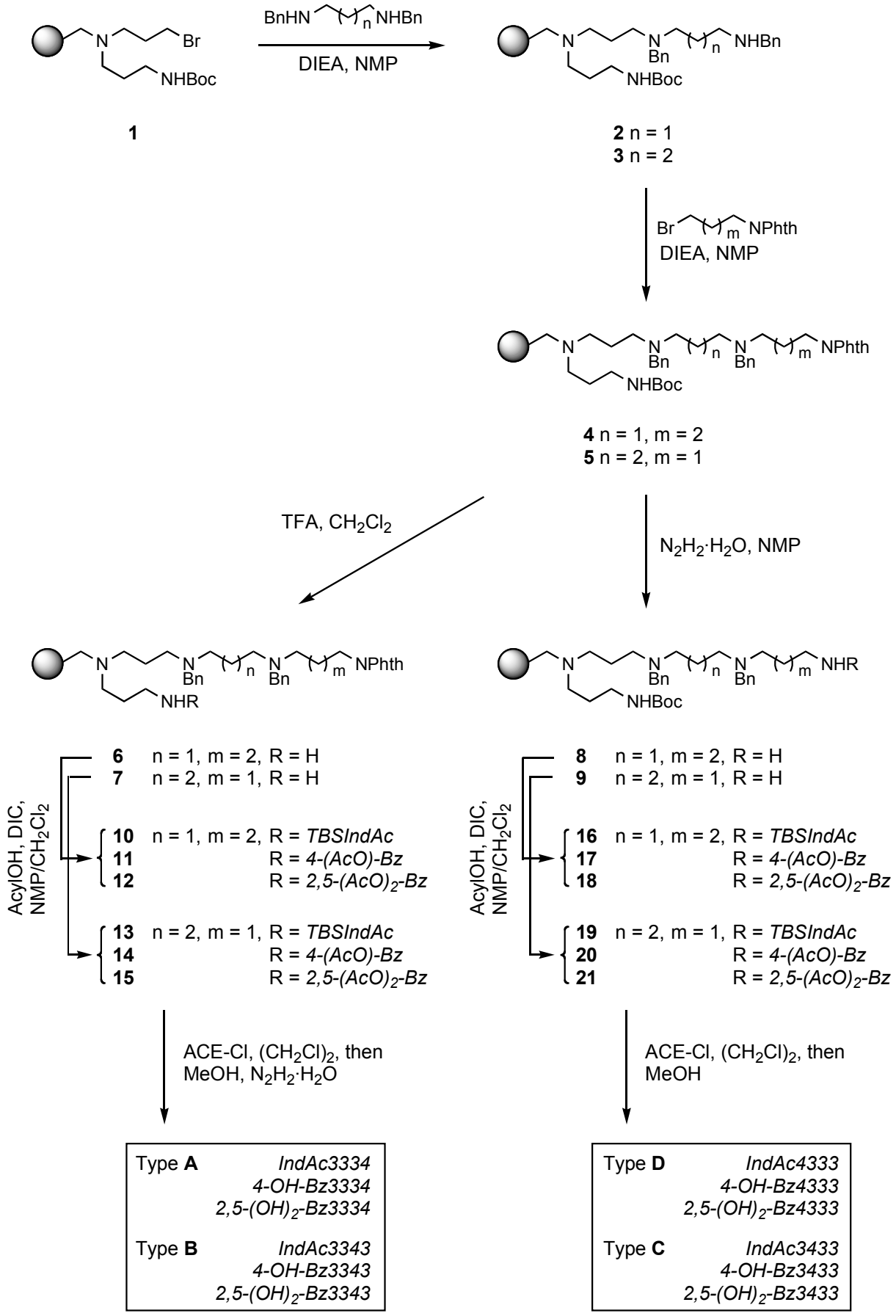
synthesized, and they were analyzed separately and in comparison with the natural compounds by HPLC-UV(DAD)-MS and -MS/MS . Additionally, the analogous substrates with the 4-hydroxybenzoic (4-OH-Bz) and the 2,5-dihydroxybenzoic (2,5-$(OH)_2$-Bz) acid head portions were prepared as well. The corresponding isomers of type **A** and **D** were found in the venom cocktail of *A. aperta*, too, but the isomeric structures of type **B** and **C** have not been identified so far. For the synthesis of the twelve target structures we had a powerful and efficient tool at hand, namely our recently developed new solid-phase methodology [9]. This methodology allows the construction of polyazaalkanes from their centers and the specific and separate modification of the linear polyamines at either end.

Results

Synthesis of the Polyamine Toxins

As already described previously, *Merrifield* resin (200–400 mesh, 1% divinylbenzene, 0.9 mmol/g loading capacity) was converted to resin **1** by its coupling with mono-Boc-protected 1,3-diamine followed by alkylation of the benzylic amine with 1,3-dibromopropane [9]. Elongation of the polyamine backbone was subsequently effected by treatment of resin **1** with *N,N′*-dibenzylpropane-1,3-diamine or *N,N′*-dibenzylbutane-1,4-diamine in 1-methyl-2-pyrrolidone (NMP) in presence of EtN(i-Pr)$_2$ and DIEA (*Scheme 1*). The resultant tetraminic resins **2** and **3** were further extended by alkylation of the terminal secondary amines with *N*-(4-bromobutyl)phthalimide or with *N*-(3-bromopropyl)phthalimide, respectively. This afforded the orthogonally protected resin-bound pentamine core structures **4** and **5**, respectively. The yields achieved so far amounted to *ca.* 30%, which was tested by removal of the polyamines from the resins and quantification of the liberated products.

To access the small library of selected target molecules, the protective groups of the terminal amino functions were cleaved off specifically and replaced with the appropriate acid moieties. Thus, resins **4** and **5** were treated either with TFA or with $N_2H_4 \cdot H_2O$ to selectively remove the Boc or phthaloyl groups, respectively. Each of the product resins **6–9** was subsequently acylated with each of the three protected acids — *N*-(*tert*-butyldimethylsilyl)-1*H*-indole-3-acetic acid (TBSIndAcOH), 4-acetoxybenzoic acid (4-(AcO)-Bz), and 2,5-diacetoxybenzoic acid (2,5-$(AcO)_2$-Bz) —



1

2 n = 1
3 n = 2

4 n = 1, m = 2
5 n = 2, m = 1

6 n = 1, m = 2, R = H
7 n = 2, m = 1, R = H

10 n = 1, m = 2, R = *TBSIndAc*
11 R = *4-(AcO)-Bz*
12 R = *2,5-(AcO)$_2$-Bz*

13 n = 2, m = 1, R = *TBSIndAc*
14 R = *4-(AcO)-Bz*
15 R = *2,5-(AcO)$_2$-Bz*

8 n = 1, m = 2, R = H
9 n = 2, m = 1, R = H

16 n = 1, m = 2, R = *TBSIndAc*
17 R = *4-(AcO)-Bz*
18 R = *2,5-(AcO)$_2$-Bz*

19 n = 2, m = 1, R = *TBSIndAc*
20 R = *4-(AcO)-Bz*
21 R = *2,5-(AcO)$_2$-Bz*

Type **A** *IndAc3334*, *4-OH-Bz3334*, *2,5-(OH)$_2$-Bz3334*
Type **B** *IndAc3343*, *4-OH-Bz3343*, *2,5-(OH)$_2$-Bz3343*

Type **D** *IndAc4333*, *4-OH-Bz4333*, *2,5-(OH)$_2$-Bz4333*
Type **C** *IndAc3433*, *4-OH-Bz3433*, *2,5-(OH)$_2$-Bz3433*

Scheme 1. *Synthetic route to the acylpentamines of type **A**–**D**.*

by their mutual treatment with diisopropyl carbodiimide (DIC). Reaction of the resins **10–21** with 1-chloroethyl chloroformate (ACE-Cl) followed by MeOH [10] or MeOH/N_2H_2·H_2O (for the *N*-phthaloyl derivatives **10–15**) provided the desired, properly derivatized twelve pentamine derivatives, devoid of all protective groups. The products were either purified by preparative HPLC or simply by rinsing with MeOH and were finally collected as their HCl or TFA salts in 10–30% overall yields.

Alternatively to the procedure described above, the phthaloyl group of the resins **10–15** can be cleaved off the polyamine prior to its release from the polymer support. Without adding N_2H_2·H_2O to the methanolic solution during the cleavage procedure, the phthaloyl group can also be retained as a protective group in the final product.

During the preparation of the indole-acetamides, we encountered one noteworthy problem. It turned out that the choice of the acid derivative to be coupled with the polyamine backbone is not trivial. Indole-3-acetic acid itself as well as its *N*-Boc-protected derivative proved not to be the appropriate reagents. Upon cleavage of the corresponding final acylpolyamines from the resin, complete decomposition of the products was observed, possibly due to oxidation. Less decomposition was noticed when more stable protective groups than Boc were used at the indole N-atom. At least, satisfactory yields of cleavage products were obtained with *N*-Bn-, *N*-Ts-, and *N*-TBDMS-protected compounds (17, 13, and 11%, respectively, for products deriving from resin **8**). Since the hydrogenolytic removal of the Bn group was accompanied with partial reduction of the indole moiety, and, also, the reductive cleavage of the Ts group by the action of MeOH/Mg was not quantitative in yield (80%), the use of the TBDMS-protected indole-acetic acid in the acylation step was finally found to be appropriate.

Correlation of the Synthetic Samples with the Natural Toxins Within the Venom

Chromatographic Behavior—Several synthetic polyamine derivatives were analyzed individually or as defined mixtures with each other and with the natural venom by HPLC-UV(DAD)-MS and HPLC-UV(DAD)-MS/MS under the standard conditions used previously [5]. *Figure 3* shows representatively the extracted-ion chromatograms (EIC) of the quasi-molecular ions at *m*/*z* 417 for the natural venom and for mixtures of the natural venom with the four synthetic *IndAc*-polyamines.

It is easily recognized from these chromatograms that the three isomeric compounds *IndAc3334*, *IndAc3343*, and *IndAc3433* all co-elute with the first fraction (t_R = 26.0

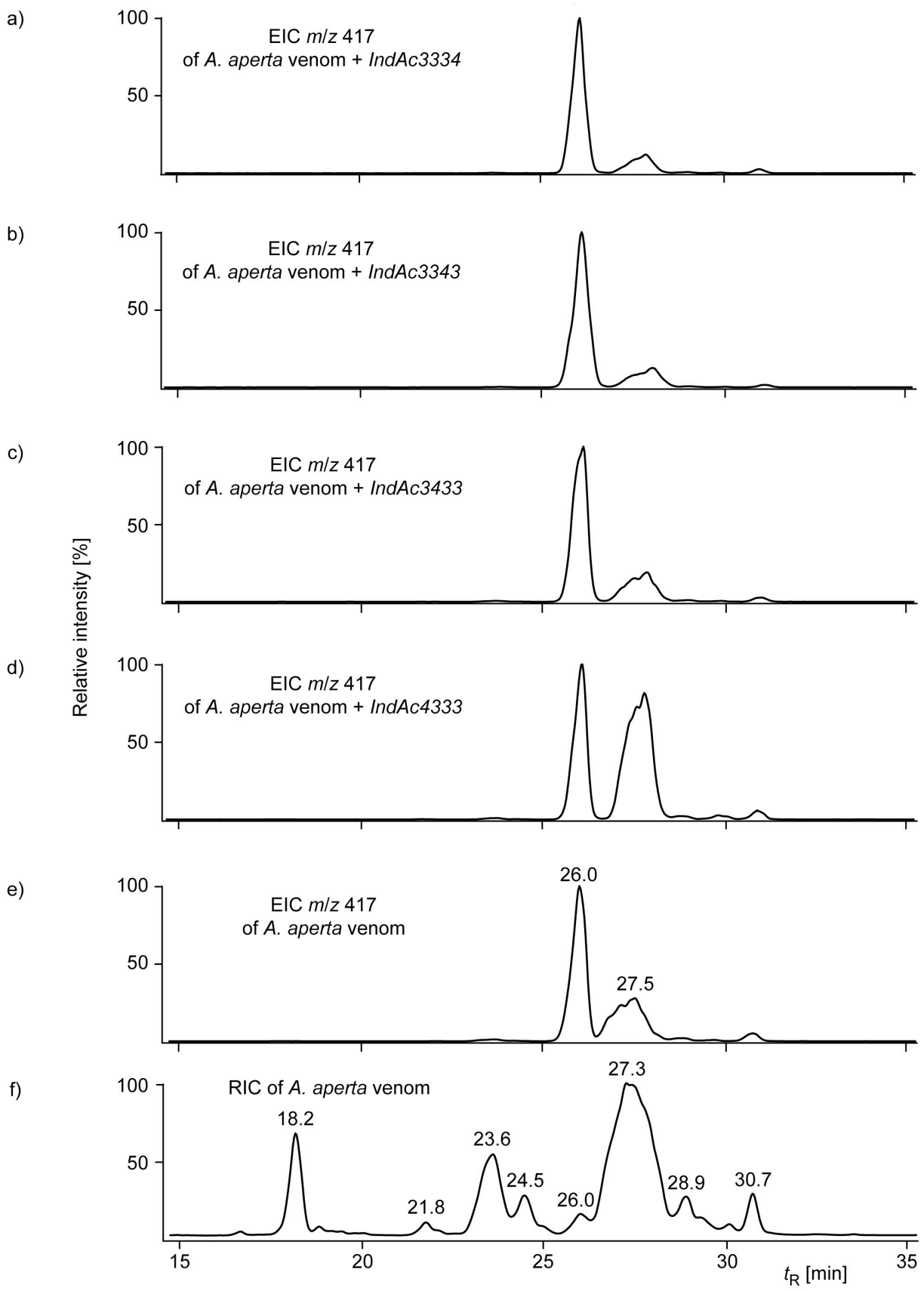


Figure 3. *Extracted ion chromatograms (EIC) detected at* m/z *417 of the venom of* A. aperta *with admixed synthetic* IndAc3334 *(a),* IndAc3343 *(b),* IndAc3433 *(c),* and IndAc4333 *(d) as well as the EIC detected at* m/z *417 (e) and the reconstructed ion chromatogram (RIC) of the natural venom (f).*

min) of the natural venom. The remaining isomer, *IndAc4333*, co-elutes with the second fraction of the native toxin mixture (t_R = 27.5 min). Similar pictures as with the *IndAc*-polyamines were gained with the other two groups of acylpolyamines (EICs of ions *m*/*z* 396 and 380 for the 4-OH-Bz and the 2,5-$(OH)_2$-Bz derivatives, respectively). Also for these compounds, the three isomers of types **A**–**C** (*Acyl3334*, *Acyl3343*, and *Acyl3433*) all co-elute with the first fractions of the natural venom components, whereas the remaining isomers of type **D** (*Acyl4333*) co-elute with the respective second fraction.

MS/MS-Fragmentation—Due to the co-elution of the several compounds in HPLC, the single-ion detected chromatographic results do not allow a definite structure correlation for all the toxins contained in the natural venom. Additional information, however, can be gained from the HPLC-MS/MS experiments. The respective spectra of the four isomeric *IndAc*-pentamines (*IndAc3334*, *IndAc3343*, *IndAc3433*, and *IndAc4333*) and of two chromatographic fractions of the natural venom are shown in *Figure 4*. The complete set of results obtained from the investigation of all synthetic polyamine derivatives and the corresponding natural toxin fractions are given in the *Experimental Part*.

It is readily realized from the illustrations in *Figure 4* that the collision induced dissociation (CID) spectra of the quasi-molecular ions of the several isomerically pure sample compounds lead to distinctively different peak patterns in the spectra. However, most of the signals (with respect to the *m*/*z* values) are shared by at least two of the four compounds. Nevertheless, characterization of the components of the natural venom by correlation with the synthetic compounds should be viable because of the distinctively different relative intensities of the signals manifested in the separate spectra. In fact, spectrum f) of the second HPLC fraction of the biological sample matches well — except for the two signals at *m*/*z* 112 and 215 — with spectrum e) of *IndAc4333*. This corroborates the earlier structure assignment for *IndAc4333*, which was recognized as the compound eluting at this position. Spectrum d) of the first HPLC fraction, on the other hand, corresponds only largely to one of the remaining spectra, namely to spectrum a) of *IndAc3334*.

The differences in the relative peak intensities and the additional ion responses found in the spectrum of the natural probe, however, are distinctive enough to deduce that *IndAc3334* cannot be the sole component detected in the venom fraction. Analysis of the corresponding spectra of the 4-OH-Bz and 2,5-$(OH)_2$-Bz polyamines

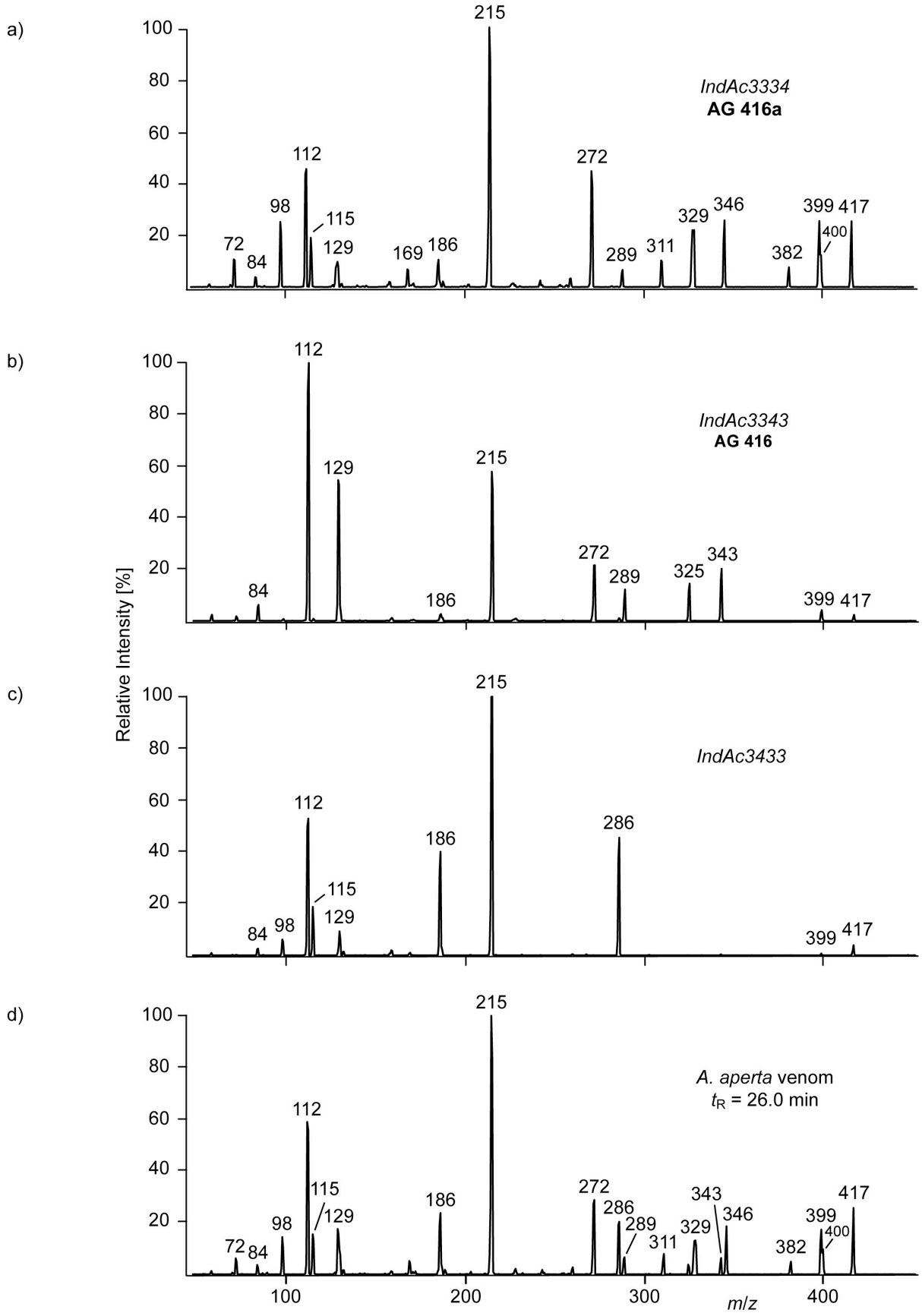


Figure 4. *MS/MS Patterns of the quasi-molecular ions at* m/z *417 of the co-eluting synthetic samples of* IndAc3334 *(a),* IndAc3343 *(b),* IndAc3433 *(c), and of the fraction with* t_R *= 26.0 min of the* A. aperta *venom (d) and the respective MS/MS of the synthetic sample of* IndAc4333 *(e) and of the fraction with* t_R *= 27.5 min of the* A. aperta *venom (f).*

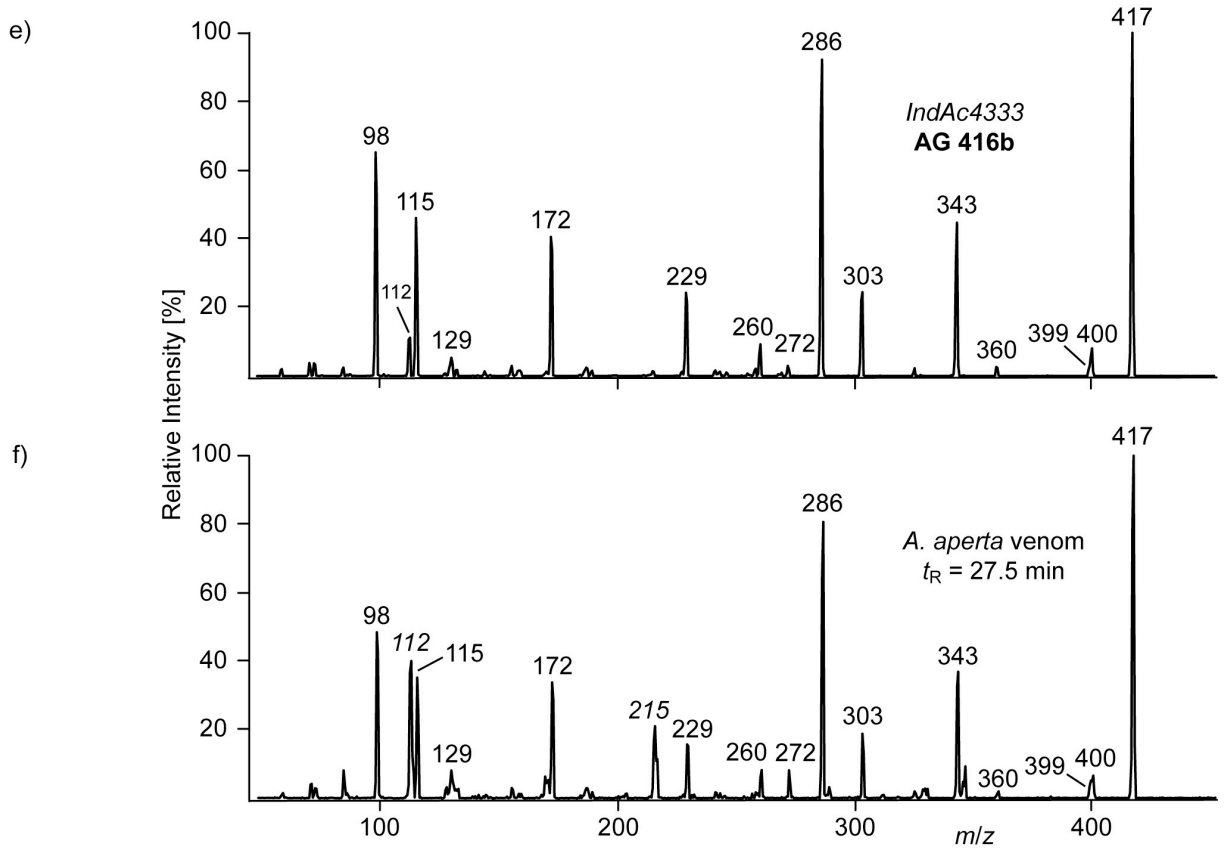
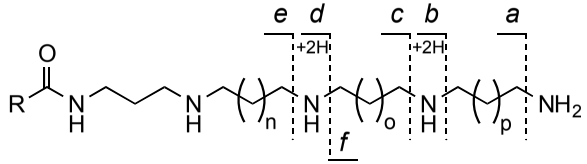


Figure 4. (continued)

revealed similar insights: the MS/MS of the second fractions of the natural toxin mixture correspond nicely to the *Acyl4333* derivatives and the spectra of the first fraction correlates largely, but not completely, to the *Acyl3334* compounds. The question arose, thus, whether it is possible to characterize one or the other of the remaining acylpolyamines as the additional constituents of the natural probe.

Scrutiny of the spectral data of the whole collection of synthetic compounds showed that only a few MS/MS signals are unique and thus characteristic for the individual isomeric acylpolyamines. Only these signals can be regarded as diagnostic for the different polyamine substructures and their attachments to the acyl groups. Of particular interest are the characteristic signals deriving from the compounds of type **A**–**C** within the three classes of isomers, because these substances co-elute in the HPLC. *Table 1* compiles these diagnostic signals obtained from the corresponding quasi-molecular ions *m*/*z* 417, 380, and 396. Additionally, some other potentially characteristic peaks and the CID patterns of the respective first eluting fractions of the natural venom are also included in *Table 1*. The data in *Table 1* shows clearly that the spectra of the first-eluting venom fractions of all three classes of acylpolyamines are each superimposable on the spectra of all three respective acylpentamines of type **A**–**C**.

Table 1. *Types and Relative Intensities of Relevant MS/MS Signals of the Quasi-Molecular Ions* m/z *417, 380, and 396 of Synthetic and Natural Acylpolyamine Samples.*



Sample		Fragments / *m/z*												
		a	*b*		*c*		*c*–H_2O		*d*		*e*		*f*	
		400	*360*	*346*	*343*	*329*	*325*	*311*	*303*	*289*	*286*	*272*	*129*	*115*
IndAc3334		13	—	26	—	22	—	10	—	7	—	45	8	– (19)
IndAc3343	*(m/z 417)*	0	0	—	20	—	14	—	—	12	– (1)	22	55	—
IndAc3433		0	0	—	0	—	0	—	0	—	46	—	3	19
Venom (t_R = 26.0)		10	0	19	7	11	4	8	0	7	21	29	18	16
	m/z	*363*	*323*	*309*	*306*	*292*	*288*	*274*	*266*	*252*	*249*	*235*	*129*	*115*
4-OH-Bz3334		9	—	11	—	19	—	7	—	11	—	34	12	– (17)
4-OH-Bz3343	*(m/z 380)*	0	0	—	11	—	4	—	—	5	—	18	46	—
4-OH-Bz3433		0	0	—	0	—	0	—	0	—	25	—	1	18
Venom (t_R = 16.7)		5	0	23	5	24	4	7	0	10	3	49	25	22
	m/z	*379*	*339*	*325*	*322*	*308*	*304*	*290*	*282*	*268*	*265*	*251*	*129*	*115*
2,5-$(OH)_2$-Bz3334		10	—	29	—	28	—	10	—	5	—	49	15	– (33)
2,5-$(OH)_2$-Bz3343	*(m/z 396)*	0	0	—	17	—	6	—	—	4	—	19	50	– (3)
2,5-$(OH)_2$-Bz3433		0	0	—	0	—	0	—	0	—	38	—	10	22
Venom (t_R = 19.5)		21	0	27	8	46	3	7	0	11	3	82	36	28

— Denotes entry positions where no fragment intensities were expected based on the structure of the sample compound, in brackets: effectively found intensities >1 rel.%; entries of 0 rel.% corresponds to the observation of no signal intensities (<1 rel.%) even though fragments would principally be expected based to the structure of the sample compound.

The major components of the natural toxin mixtures are, in all cases, recognized as the *Acyl3334* derivatives, as already realized above for *IndAc3334*. All four characteristic peaks for the fragments of type *a*, *b*, *c*, and *c*–H_2O of these isomers are found in high abundance in the three spectra of the natural compound fractions. The presence of the *Acyl3343* derivatives in the natural mixture is manifested with the two diagnostic fragments of type *c* and *c*–H_2O, which were recorded with substantial intensities in all three spectra of the natural venom fractions. For the *Acyl3433* derivatives, only one single characteristic signal, corresponding to a fragment of type *e*, can be extracted from the data of the isomerically pure samples, and the respective responses for fragment *e* are detected also in the spectra of the natural venom fractions. While the abundance of fragment *e* for *IndAc3433* is substantial in the spectrum of the venom (21 rel.%), the intensities for the related fragments of *4-OH-Bz3433* and *2,5-(OH)$_2$-Bz3433* are rather low (3 rel.% each). Nevertheless, these signals are regarded as significant enough to conclude that all three *Acyl3433* derivatives are constituents of the natural spider venom.

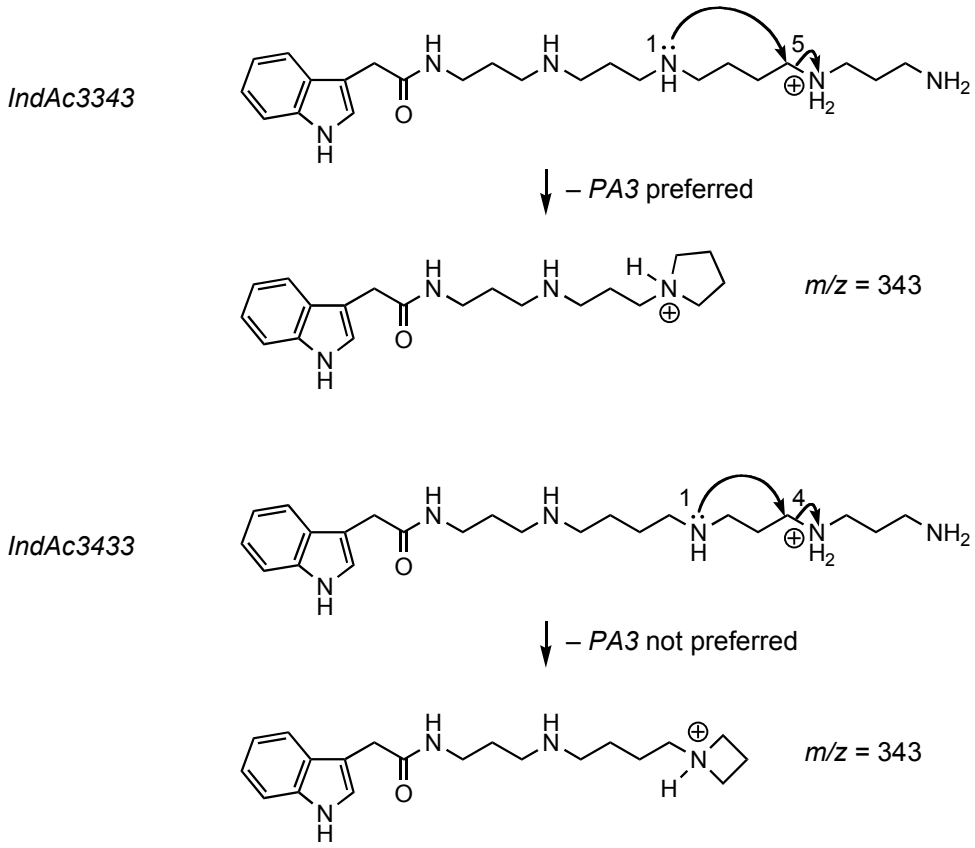
Discussion

Our comparative HPLC-MS/MS investigation of synthetic polyamine derivatives and of natural polyamine fractions of *A. aperta* largely supports the results and deductions published earlier [5]. It unambiguously confirms all the previously proposed compounds to be contained in the natural venom. It furthermore establishes the occurence of five additional polyamine toxins that have not been identified so far. We have shown that the venom contains, for each of the three aromatic head groups, all four possible isomeric acylpolyamines related to the parent pentamines *PA3334* and *PA3343*, *i.e.*, all compounds of type **A**–**D**.

The presence of all these isomeric acylpentamines is strongly indicative for the biosynthetic pathway of the compounds in spiders. Even though the free polyamines themselves were not yet reported in the venom of *A. aperta*, we assume that they represent the biosynthetic precursors of the several toxins. We propose that the final toxin cocktail of the spiders is derived from 'statistical' acylation of the two parent pentamines at either end of the molecules.

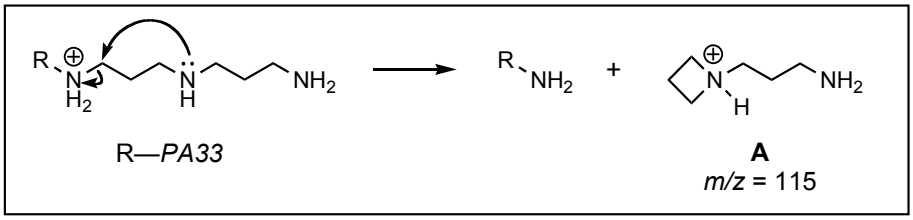
Besides the identification of the twelve synthetic acylpentamines as constituents of the venom of *A. aperta*, our MS/MS study exposed also some interesting spectrometric peculiarities. First, the spectra of the synthetic *IndAc3343* and

IndAc3433 revealed that the signal at *m*/*z* 343 for fragment *c* is, in fact, indicative for the former compound. As outlined earlier, the formation of a fragment with *m*/*z* 343 could principally also be due to the decomposition of *IndAc3433*. However, the corresponding fragmentation of the latter is much less pronounced. This is possible because the reaction, in contrast to the reaction of *IndAc3343*, cannot proceed *via* a five-membered cyclic intermediate. The respective proposed fragmentation reactions are outlined in *Scheme 2*. The same effect as with *IndAc3343* and *IndAc3433* was observed for the two other pairs of acylpolyamines with the 4-OH-Bz and the 2,5-$(OH)_2$-Bz groups (fragments *c* at *m*/*z* 306 and 325, respectively).

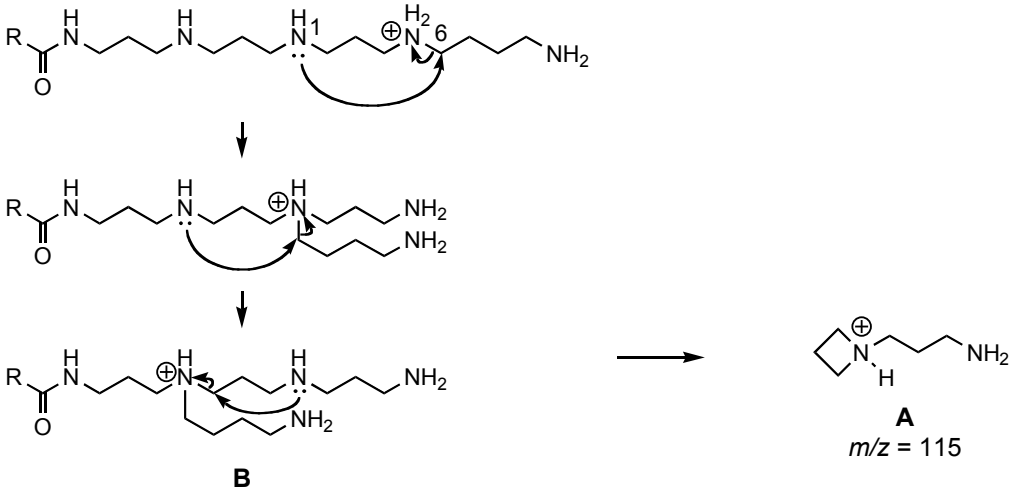


Scheme 2. *Proposed fragmentation reactions leading to the formation of fragment ions at* m/z *343 from* IndAc3343 *and* IndAc3433.

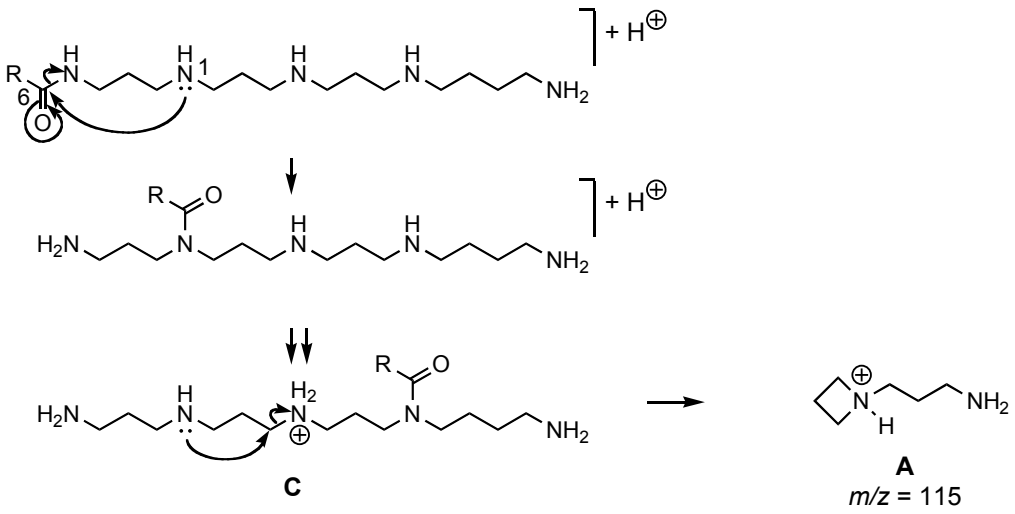
It is evidently also not restricted to the formation of fragments of type *c*: all fragmentations proceeding analogously *via* five-membered cyclic transition states are clearly favored reactions. This is demonstrated, for instance, with the abundant signals for fragments *a* observed for the *Acyl3334* derivatives. They are due to the loss of NH_3 from the respective quasi-molecular ions by intramolecular nucleophilic substitutions (s_Ni) *via* five-membered transition states. The analogous reaction of the isomeric acylpolyamines would all have to proceed *via* four-membered transition



ZIP-reaction by transamination



ZIP-reaction by transamidation



Scheme 3. *Proposed gas-phase* Zip*-reactions by transamination or transamidation of the parent* Acyl3334 *derivatives leading to the formation of the fragment ion* ***A*** *at* m/z *115.*

structures and are, thus, less pronounced; in fact, the respective signals are not observed at all.

Another peculiarity in the fragmentation behavior of the acylpolyamines became evident. We would have expected, as mentioned earlier, to find fragment *f* at *m*/*z* 115 to be decisive for the *Acyl3433* derivatives (*Scheme 3*). The fragment, which was attributed to structure **A**, would be released from a polyamine with a terminal *PA33* unit. Fragment *f* at *m*/*z* 115, however, was also observed with high abundance in the MS/MS of the *Acyl3334* derivatives lacking the *PA33* termini. It was not found, or was found only with negligible intensity, however, in the *Acyl3343* derivatives possessing an inverted *PA34* unit at the end. The formation of the fragment with *m*/*z* 115 from *Acyl3334* derivatives can be explained by two processes, which involve a *ZIP*-reaction (*Scheme 3*) [11]. The quasi-molecular ions could lead either by a cascade of s_Ni reactions (transaminations) or transamidation processes — all proceeding *via* six-membered cyclic transition states — to intermediary structures **B** or **C**. These intermediates are both prone to loose fragment **A** corresponding to the observed signal. The analogous *ZIP*-reactions with the *Acyl3343* derivatives are less advantageous since, they would involve at one stage a group transfer *via* an unfavorable seven-membered cyclic transition state [12-14]. Acid-catalyzed transamidation during MS analysis was already found earlier [15]; for the transamination reaction, no literature precedence is available. The latter reaction was nevertheless considered because relevant and expected fragmentation of the transamidation products was not observed. For instance, no loss of *PA33* from ion **C** was detected (*m*/*z* 286, 249, and 265, respectively, for the three *Acyl3334*).

Conclusion

Our study has shown that the conclusive and final analysis of trace components within complex mixtures of polyamine spider toxins needs synthetic reference samples of known structures. With such compounds at hand, we were not only able to confirm seven proposed structures found previously in the venom of *A. aperta* but also to detect five additional acylpolyamines, completing the set of isomeric constituents contained in the natural sample. The solid-phase synthesis of the polyamine derivatives proved to be a viable way to rapidly prepare the small library of spider toxins, and we are confident that this strategy can be extended for the preparation of more complex molecules as well.

Experimental Part

1. *General*. Unless otherwise stated, starting materials were obtained from commercial suppliers and were used without further purification. Lyophilized *Agelenopsis aperta* venom was purchased from *Spider Pharm. Inc.*, Yarnell, AZ, U.S.A. and was stored at –80°. As the solid support, *Merrifield* peptide resin 200–400 mesh, 1% divinylbenzene, loading 0.9 mmol/g from *Advanced ChemTech* was used. Instrumentation for the solid phase reactions: *PLS 4×6* and *PLS 1×6 Organic Synthesizers*. IR spectra: as KBr presslings; *Perkin-Elmer IR 'Spectrum One'* and *Perkin-Elmer* 781; in cm^{-1}. ^{1}H-NMR spectra: D_2O or $CDCl_3$; *Bruker AC-300* (300 MHz); δ in ppm rel. to TSP (δ 0.00) or $CHCl_3$ (δ 7.26), *J* in Hz. ^{13}C NMR spectra: D_2O or $CDCl_3$; *Bruker ARX-300* (75.5 MHz); δ in ppm rel. to TSP (δ 1.7) or $CDCl_3$ (δ 77.0); multiplicities from DEPT-135 and DEPT-90 experiments. HPLC-UV(DAD)-MS and HPLC-UV(DAD)-MS/MS: see detailed description under paragraph *7*. Preparative chromatographic conditions (HPLC): columns *Kromasil KR100–10C18* (4.6 × 250 mm) and *Kromasil KR100–10C18* (50.8 × 250 mm); H_2O was purified with a *Milli-*Q_{RG} apparatus. Confirmation of structures and purities of the final polyamine derivatives are provided by their ^{1}H-NMR spectra and by their HPLC-MS analysis. Elemental analyses and HR-MS is not appropriate for polyamine derivatives since the compounds arise, as free bases, as waxy or glassy solids, from which the last solvent molecules can hardly be removed. The HCl salts are rather hygroscopic, and the uptake of H_2O falsifies the elemental analyses. The compounds are not stable enough to survive distillation and show heavy fragmentation in EI-MS. HR-MS on the molecular ions is thus not possible, and HR-MS on fragment ions are not informative enough to establish the overall structures.

2. *Construction of the Polyamine Backbones on the Resin.* 2.1. *Elongation of Resin* **1** *with* N,N'-*Dibenzylpropane-1,3-diamine (→ Resin* **2***)*. Resin **1** (4.1 mmol [9]) was swelled in 1-methylpyrrolidin-2-one (NMP; 30 ml). *N,N'*-Dibenzylpropane-1,3-diamine (6.25 g, 24.6 mmol [16]) and $EtN(i\text{-}Pr)_2$ (DIEA; 7.02 ml, 41 mmol) were added. After agitation for 24 h at 50°, resin **2** was filtered off, washed with NMP and CH_2Cl_2, and dried *in vacuo*.

2.2. *Elongation of Resin* **1** *with* N,N'-*Dibenzylbutane-1,4-diamine (→ Resin* **3***)*. Resin **1** (4.1 mmol [9]) was swelled in NMP (30 ml), and *N,N'*-dibenzylbutane-1,4-diamine (6.59

g, 24.6 mmol) and DIEA (7.02 ml, 41 mmol) were added. After agitation for 24 h at 50°, resin **3** was filtered off, washed with NMP and CH_2Cl_2, and dried *in vacuo*.

2.3. *Alkylation of Resin* **2** *with* N-*(4-Bromobutyl)phthalimide (→ Resin* **4***).* Resin **2** (4.1 mmol) was suspended in NMP (30 ml). *N*-(4-Bromobutyl)phthalimide (5.784 g, 20.5 mmol) and DIEA (7.02 ml, 41 mmol) were added, and the mixture was agitated for 26 h at 50°. Resin **4** was filtered off, washed with NMP, CH_2Cl_2 and MeOH, and dried *in vacuo*. IR: 3426*w*, 1772*w*, 1714*s*.

2.4. *Alkylation of Resin* **3** *with* N-*(3-Bromopropyl)phthalimide (→ Resin* **5***).* Resin **3** (4.1 mmol) was suspended in NMP (30 ml). *N*-(3-Bromopropyl)phthalimide (5.494 g, 20.5 mmol) and DIEA (7.02 ml, 41 mmol) were added and the mixture was agitated for 26 h at 50°. Resin **5** was filtered off, washed with NMP, CH_2Cl_2 and MeOH, and dried *in vacuo*. IR: 3427*w*, 1772*m*, 1714*s*.

3. *Deprotection of the Terminal Amino Groups of the Resins.* 3.1. *Removal of the Boc Group from Resin* **4** *(→ Resin* **6***).* To resin **4** (1.64 mmol), swelled in CH_2Cl_2 (16 ml) was added CF_3COOH (TFA; 4.0 ml, 52 mmol). After agitation for 15 h at 23°, resin **6** was filtered off, washed with CH_2Cl_2, CH_2Cl_2/DIEA 3:1, NMP, and CH_2Cl_2, and dried *in vacuo* at 50°. IR: 3384*w*, 1771*w*, 1714*s*.

3.2. *Removal of the Boc Group from Resin* **5** *(→ Resin* **7***).* To resin **5** (1.64 mmol), swelled in CH_2Cl_2 (16 ml) was added TFA (4.0 ml, 52 mmol). After agitation for 15 h at 23°, resin **7** was filtered off, washed with CH_2Cl_2, CH_2Cl_2/DIEA 3:1, NMP, and CH_2Cl_2, and dried *in vacuo* at 50°. IR: 3420*w*, 1771*w*, 1714*s*.

3.3. *Removal of the Phthaloyl Group from Resin* **4** *(→ Resin* **8***).* Resin **4** (1.64 mmol) was swelled in NMP (15 ml), and the mixture, after addition of $N_2H_4 \cdot H_2O$ (6.0 ml, 0.123 mol), was agitated for 3 h at 80°. Resin **8** was filtered off, washed with NMP, dioxane, dioxane/H_2O 1:1, dioxane, and CH_2Cl_2, and dried *in vacuo* at 50°. IR: 3426*w*, 1714*s*.

3.4. *Removal of the Phthaloyl Group from Resin* **5** *(→ Resin* **9***).* Resin **5** (1.64 mmol) was swelled in NMP (15 ml) and the mixture, after addition of $N_2H_4 \cdot H_2O$ (6.0 ml, 0.123 mol), was agitated for 3 h at 80°. Resin **9** was filtered off, washed with NMP, dioxane, dioxane/H_2O 1:1, dioxane, and CH_2Cl_2, and dried *in vacuo* at 50°. IR: 3427*w*, 1714*s*.

4. *Acylation of the Resins.* 4.1. *General Procedure (GP 4.1).* The resin (1.64 mmol) was swelled in NMP/CH_2Cl_2 1:2 (15 ml). The appropriate carboxylic acid (16.4 mmol) and *N*,*N'*-diisopropylcarbodiimide (1.27 ml, 8.2 mmol) were added, and the mixture was agitated for 30 h at 23°. The product resin was filtered off, washed successively with CH_2Cl_2, NMP, NMP/DIEA 10:1, NMP, and CH_2Cl_2, and was dried *in vacuo* at 50°. The *Kaiser* test [17] was performed to prove the absence of primary amino groups.

4.2. *Acylation of Resin* **6** *with TBSIndAcOH (→ Resin* **10**). Resin **6** was acylated with 2-{1-[(*tert*-butyl)dimethylsilyl]-1*H*-indole-3}-acetic acid according to *GP 4.1* to give resin **10**. IR: 3420*m*, 1770*w*, 1710*s*, 1665*s*.

4.3. *Acylation of Resin* **6** *with 4-(AcO)-Bz (→ Resin* **11**). Resin **6** was acylated with 4-acetoxybenzoic acid according to *GP 4.1* to give resin **11**. IR: 3420*m*, 1760*m*, 1715*s*, 1660*m*.

4.4. *Acylation of Resin* **6** *with 2,5-$(AcO)_2$-Bz (→ Resin* **12**). Resin **6** was acylated with 2,5-diacetoxybenzoic acid according to *GP 4.1* to give resin **12**. IR: 3420*w*, 1765*s*, 1710*m*, 1665*m*.

4.5. *Acylation of Resin* **7** *with TBSIndAcOH (→ Resin* **13**). Resin **7** was acylated with 2-{1-[(*tert*-butyl)dimethylsilyl]-1*H*-indole-3}-acetic acid according to *GP 4.1* to give resin **13**. IR: 3420*w*, 1775*w*, 1715*s*, 1680*s*.

4.6. *Acylation of Resin* **7** *with 4-(AcO)-Bz (→ Resin* **14**). Resin **7** was acylated with 4-acetoxybenzoic acid according to *GP 4.1* to give resin **14**. IR: 3420*m*, 1760*m*, 1715*s*, 1660*m*.

4.7. *Acylation of Resin* **7** *with 2,5-$(AcO)_2$-Bz (→ Resin* **15**). Resin **7** was acylated with 2,5-diacetoxybenzoic acid according to *GP 4.1* to give resin **15**. IR: 3420*w*, 1765*s*, 1710*m*, 1665*m*.

4.8. *Acylation of Resin* **8** *with TBSIndAcOH (→ Resin* **16**). Resin **8** was acylated with 2-{1-[(*tert*-butyl)dimethylsilyl]-1*H*-indole-3-}acetic acid according to *GP 4.1* to give resin **16**. IR: 3420*w*, 1710*s*, 1690*s*.

4.9. *Acylation of Resin* **8** *with 4-(AcO)-Bz (→ Resin* **17**). Resin **8** was acylated with 4-acetoxybenzoic acid according to *GP 4.1* to give resin **17**. IR: 3425*w*, 1760*m*, 1714*s*, 1663*m*.

4.10. *Acylation of Resin* **8** *with 2,5-(AcO)$_2$-Bz (→ Resin* **18**). Resin **8** was acylated with 2,5-diacetoxybenzoic acid according to *GP 4.1* to give resin **18**. IR: 3420*w*, 1765*m*, 1715*s*, 1650*m*.

4.11. *Acylation of Resin* **9** *with TBSIndAcOH (→ Resin* **19**). Resin **9** was acylated with 2-{1-[(*tert*-butyl)dimethylsilyl]-1*H*-indole-3}-acetic acid according to *GP 4.1* to give resin **13**. IR: 3420*w*, 1710*s*, 1690*s*.

4.12. *Acylation of Resin* **9** *with 4-(AcO)-Bz (→ Resin* **20**). Resin **9** was acylated with 4-acetoxybenzoic acid according to *GP 4.1* to give resin **20**. IR: 3425*m*, 1760*m*, 1715*s*, 1640*m*.

4.13. *Acylation of Resin* **9** *with 2,5-(AcO)$_2$-Bz (→ Resin* **21**). Resin **9** was acylated with 2,5-diacetoxybenzoic acid according to *GP 4.1* to give resin **21**. IR: 3420*w*, 1765*m*, 1715*s*, 1650*m*.

5. *Cleavage of the Polyamine Derivatives from the Resins. 5.1. General Procedure (GP 5.1).* The resin (1.64 mmol) was swelled in 1,2-dichloroethane (15 ml) and 1-chloroethyl chloroformate (ACE-Cl; 3.57 ml, 32.8 mmol) was added. After agitation for 3 h at 23°, the product resin was filtered off and washed with CH_2Cl_2. The org. solns. were combined and evaporated to dryness. The residues were dissolved in MeOH, and the resulting soln. was refluxed for 3 h. For resins **10**–**15** (Phth-protected compounds), $N_2H_2{\cdot}H_2O$ (2.0 ml, 41 mmol) were added, and refluxing was continued for an additional 2 h. The solvent was removed, and the solid residue, after washing of the residue with MeOH, was collected. This provided the corresponding polyamine derivative as the 4 HCl salt. Where appropriate, purification was followed by HPLC.

5.2. N-*(16-Amino-4,8,12-triazahexadecyl)-1*H-*indole-3-acetamide* (*IndAc3334*, **AG 416a**). Treatment of **10** (1.55 mmol) according to *GP 5.1* afforded *IndAc3334*·4 HCl (115 mg, 0.20 mmol; overall 13% with respect to **1**) after purification by HPLC (solvent A: 0.05% HCl in MeOH; solvent B: 0.05% HCl in H_2O; 25% A; λ = 254 nm; flow rate 20 ml min^{-1}; the product was collected at 18.2–20.8 min). IR: 3380*s*, 3320*m*, 2950*s*, 2750*s*, 2520*m*, 2405*m*, 1650*s*, 1610*w*, 1535*m*, 1455*m*, 1050*w*, 780*w*, 740*m*. ^{1}H-NMR (D_2O): 7.68–7.53 (*m*, 2 arom. H); 7.38–7.15 (*m*, 3 arom. H); 3.77 (*s*, ArCH_2); 3.35–3.00 (*m*, 12 H); 2.98–2.82 (*m*, 4 H); 2.22–1.94 (*m*, 4 H); 1.91–1.70 (*m*, 6 H). ^{13}C-NMR (D_2O): 180.5 (*s*, CO); 140.8, 131.2 (2*s*, 2 arom. C); 129.7, 126.7, 124.1, 122.8, 116.6 (5*d*, 5 arom. C); 112.2

(*s*, 1 arom. C); 51.6, 49.6, 49.17, 49.16, 49.1, 48.9, 43.4, 40.5, 37.0, 30.1, 28.4, 27.3, 27.2, 27.1 (14*t*). MS see paragraph 7.

5.3. N-*(16-Amino-4,8,12-triazahexadecyl)-4-hydroxybenzamide* (*4-OH-Bz3334*, **AG 379**). Treatment of **11** (1.62 mmol) according to *GP 5.1* afforded *4-OH-Bz3334*·4 HCl (250 mg, 0.48 mmol; overall 30% with respect to **1**) after purification by HPLC (solvent 0.05% HCl in H_2O; λ = 254 nm; flow rate 25 ml min^{-1}; the product was collected at 31.5–41.0 min). IR: 3400*s*, 2955*s*, 2770*s*, 2550*s*, 2348*w*, 1635*s*, 1610*s*, 1505*s*, 1255*m*, 1088*s*, 1058*s*, 922*m*. ^{1}H-NMR (D_2O): 7.69 (*d*, *J* = 8.9, 2 arom. H); 6.97 (*d*, *J* = 8.9, 2 arom. H); 3.48 (*t*, *J* = 6.7, 2 H); 3.27–3.02 (*m*, 14 H); 2.24–1.95 (*m*, 6 H); 1.84–1.72 (*m*, 4 H). ^{13}C-NMR (D_2O): 174.9 (*s*, CO); 163.5 (*s*, 1 arom. C); 133.7 (*d*, 2 arom. C); 129.2 (*s*, 1 arom. C); 119.8 (*d*, 2 arom. C); 51.4 (*t*, 2 C); 49.9 (*t*); 49.0 (*t*, 2 C); 48.8, 42.9, 40.9, 30.0, 28.4, 27.1 (6*t*); 27.0 (*t*, 2 C). MS see paragraph 7.

5.4. N-*(16-Amino-4,8,12-triazahexadecyl)-2,5-dihydroxybenzamide* (*2,5-$(OH)_2$-Bz3334*, **AG 395b**). Treatment of **12** (1.62 mmol) according to *GP 5.1* afforded *2,5-$(OH)_2$-Bz3334*·4 TFA (281 mg, 0.33 mmol; overall 20% with respect to **1**) after purification by HPLC (solvent A: 0.05% TFA in MeOH; solvent B: 0.05% TFA in H_2O; 11% A; λ = 240 nm; flow rate 25 ml min^{-1}; the product was collected at 41.0–62.0 min). IR: 3360*m*, 3095*m*, 2880*m*, 1670*s*, 1605*m*, 1490*m*, 1425*m*, 1200*s*, 1165*s*, 1130*s*, 835*m*, 800*m*, 720*s*. ^{1}H-NMR (D_2O): 7.10 (*d*, *J* = 3.1, 1 arom. H); 6.92 (*dd*, *J* = 3.1, 8.8, 1 arom. H); 6.80 (*d*, *J* = 8.8, 1 arom. H); 3.43 (*t*, *J* = 6.6, 2 H); 3.19–2.93 (*m*, 14 H); 2.15–1.88 (*m*, 6 H); 1.80–1.61 (*m*, 4 H). ^{13}C-NMR (D_2O): 174.1 (*s*, CONH); 167.1 (*q*, *J* = 36, 4 CO(TFA)); 154.9, 152.9 (2*s*, 2 arom. C); 125.9, 122.7 (2*d*, 2 arom. C); 121.3 (*s*, 1 arom. C); 120.7 (*q*, *J* = 291, 4 CF_3); 118.6 (*d*, 1 arom. C); 51.5, 49.7 (2*t*); 49.0, 48.8 (2*t*, 2×2 C); 43.2, 40.6, 30.1, 28.3, 27.2 (5*t*); 27.0 (*t*, 2 C). MS see paragraph 7.

5.5. N-*(16-Amino-4,8,13-triazahexadecyl)-1*H-*indole-3-acetamide* (*IndAc3343*, **AG 416**). Treatment of **13** (0.80 mmol) according to *GP 5.1* afforded *IndAc3343*·4 HCl (45 mg, 0.08 mmol; overall 10% with respect to **1**) after purification by HPLC (solvent A: 0.05% HCl in MeOH; solvent B: 0.05% HCl in H_2O; 25% A; λ = 254 nm; flow rate 20 ml min^{-1}; the product was collected at 17.9–19.5 min). IR: 3380*s*, 3325*m*, 2950*s*, 2750*s*, 2520*m*, 2405*w*, 1650*s*, 1535*m*, 1455*m*, 1060*w*, 780*w*, 740*m*. ^{1}H-NMR (D_2O): 7.67–7.49 (*m*, 2 arom. H); 7.38–7.15 (*m*, 3 arom. H); 3.76 (*s*, ArCH_2); 3.28 (*t*, *J* = 6.5, NCH_2); 3.23–3.01 (*m*, 10 H); 2.97–2.82 (*m*, 4 H); 2.19–1.92 (*m*, 4 H); 1.89–1.73 (*m*, 6 H). ^{13}C-NMR (D_2O):

180.5 (*s*, CO); 140.8, 131.1 (2*s*, 2 arom. C); 129.7, 126.7, 124.1, 122.8, 116.6 (5*d*, 5 arom. C); 112.2 (*s*, 1arom. C); 51.5 (*t*, 2 C); 49.6, 49.1 (2*t*); 48.9 (*t*, 2 C); 41.1, 40.4, 37.0, 30.1, 28.3 (5*t*); 27.3 (*t*, 2 C); 27.1 (*t*). MS see paragraph 7.

5.6. N-*(16-Amino-4,8,13-triazahexadecyl)-4-hydroxybenzamide* (*4-OH-Bz3343).* Treatment of **14** (1.62 mmol) according to *GP 5.1* afforded *4-OH-Bz3343*·4 HCl (100 mg, 0.19 mmol; overall 12% with respect to **1**) after purification by HPLC (solvent 0.06% HCl in H_2O; λ = 254 nm; flow rate 20 ml min^{-1}; the product was collected at 42.5–49.0 min). IR: 3320*m*, 2950*s*, 2780*s*, 1640*m*, 1610*m*, 1510*m*, 1460*m*, 845*m*. ^{1}H-NMR (D_2O): 7.70 (*d*, *J* = 8.8, 2 arom. H); 6.96 (*d*, *J* = 8.8, 2 arom. H); 3.48 (*t*, *J* = 6.6, 2 H); 3.22–3.06 (*m*, 14 H); 2.22–1.94 (*m*, 6 H); 1.86–1.70 (*m*, 4 H). ^{13}C-NMR (D_2O): 175.2 (*s*, CO); 163.8 (*s*, 1 arom. C); 133.9 (*d*, 2 arom. C); 129.6 (*s*, 1 arom. C); 120.0 (*d*, 2 arom. C); 51.5 (*t*, 2 C); 49.8, 49.1 (2*t*); 49.0 (*t*, 2 C); 41.1, 41.0, 30.3, 28.3 (4*t*); 27.3 (*t*, 2 C); 27.2 (*t*). MS see paragraph 7.

5.7. N-*(16-Amino-4,8,13-triazahexadecyl)-2,5-dihydroxybenzamide* (*2,5-$(OH)_2$-Bz3343*). Treatment of **15** (1.62 mmol) according to *GP 5.1* afforded *2,5-$(OH)_2$-Bz3343*·4 HCl (110 mg, 0.20 mmol; overall 12% with respect to **1**) after purification by HPLC (solvent A: 0.06% HCl in MeOH; solvent B: 0.06% HCl in H_2O; 11% A; λ = 254 nm; flow rate 20 ml min^{-1}; the product was collected at 17.0–21.0 min). IR: 3425*m*, 3360*m*, 2950*s*, 2750*s*, 1645*w*, 1600*m*, 1490*s*, 1230*m*, 820*m*, 790*w*. ^{1}H-NMR (D_2O): 7.09 (*d*, *J* = 3.0, 1 arom. H); 6.93 (*dd*, *J* = 3.0, 8.9, 1 arom. H); 6.82 (*d*, *J* = 8.9, 1 arom. H); 3.46 (*t*, *J* = 6.7, 2H); 3.23–3.04 (*m*, 14 H); 2.23–1.95 (*m*, 6 H); 1.84–1.74 (*m*, 4 H). ^{13}C-NMR (D_2O): 174.0 (*s*, CO); 155.2, 152.8 (2*s*, 2 arom. C); 126.0, 122.7 (2*d*, 2 arom. C); 121.4 (*s*, 1 arom. C); 118.5 (*d*, 1 arom. C); 51.5 (*t*, 2 C); 49.9, 49.10, 49.06, 49.0, 41.2, 40.8, 30.2, 29.3 (8*t*); 27.3 (*t*, 2 C); 27.2 (*t*). MS see paragraph 7.

5.8. N-*(16-Amino-4,9,13-triazahexadecyl)-1*H-*indole-3-acetamide* (*IndAc3433*). Treatment of **19** (1.55 mmol) according to *GP 5.1* afforded *IndAc3433*·4 HCl (105 mg, 0.19 mmol; overall 12% with respect to **1**) after purification by HPLC (solvent A: 0.05% HCl in MeOH; solvent B: 0.05% HCl in H_2O; 12.5% A; λ = 220 nm; flow rate 20 ml min^{-1}; the product was collected at 25.5–36.5 min). IR: 3400*s*, 3300*m*, 2950*s*, 2750*s*, 2490*m*, 1650*m*, 1610*w*, 1455*m*, 740*m*.^{1}H-NMR (D_2O): 7.67–7.47 (*m*, 2 arom. H); 7.38–7.16 (*m*, 3 arom. H); 3.77 (*s*, ArCH_2); 3.34–2.65 (*m*, 16 H); 2.22–2.07 (*m*, 4 H); 1.89–1.54 (*m*, 6 H). ^{13}C-NMR (D_2O): 180.5 (*s*, CO); 140.9, 131.1 (2*s*, 2 arom. C); 129.7, 126.7, 124.1, 122.9, 116.6

(5*d*, 5 arom. C); 112.3 (*s*, 1 arom. C); 51.5, 51.3, 49.4, 49.3, 49.2, 49.0, 41.1, 40.5, 37.0, 30.1, 28.3 (11*t*); 27.2 (*t*, 2 C); 27.1 (*t*). MS see paragraph 7.

5.9. N-*(16-Amino-4,9,13-triazahexadecyl)-4-hydroxybenzamide* (*4-OH-Bz3433*). Treatment of **20** (1.62 mmol) according to *GP 5.1* afforded *4-OH-Bz3433*·4 TFA (159 mg, 0.2 mmol; overall 12% with respect to **1**) after purification by HPLC (solvent A: 0.05% TFA in MeOH; solvent B: 0.05% TFA in H_2O; 0–65 min 5% A; 65–100 min 20% A; λ = 254 nm; flow rate 20 ml min^{-1}; the product was collected at 83.5–89.1 min). IR: 3400*m*, 3100*m*, 2880*m*, 1670*s*, 1605*m*, 1510*m*, 1475*m*, 1425*m*, 1200*s*, 1160*s*, 1130*s*, 835*m*, 800*m*, 720*s*.^{1}H-NMR (D_2O): 7.61 (*d*, *J* = 8.8, 2 arom. H); 6.88 (*d*, *J* = 8.8, 2 arom. H); 3.40 (*t*, *J* = 6.6, 2 H); 3.16–2.97 (*m*, 14 H); 2.18–1.86 (*m*, 6 H); 1.78–1.66 (*m*, 4 H). ^{13}C-NMR (D_2O): 175.0 (*s*, CONH); 167.1 (*q*, *J* = 36, 4 CO(TFA)); 163.7 (*s*, 1 arom. C); 133.8 (*d*, 2 arom. C); 129.5 (*s*, 1 arom. C); 120.7 (*q*, *J* = 291, 4 CF_3); 119.9 (*d*, 2 arom. C); 51.4, 51.3, 49.6, 49.1, 49.0, 48.8, 41.0, 40.9, 30.2, 28.2 (10*t*); 27.2 (*t*, 2 C); 27.1 (*t*). MS see paragraph 7.

5.10. N-*(16-Amino-4,9,13-triazahexadecyl)-2,5-dihydroxybenzamide* (*2,5-$(OH)_2$-Bz3433*). Treatment of **21** (1.64 mmol) according to *GP 5.1* afforded *2,5-$(OH)_2$-Bz3433*·4 TFA (182 mg, 0.21 mmol; overall 13% with respect to **1**) after purification by HPLC (solvent A: 0.05% TFA in MeOH; solvent B: 0.05% TFA in H_2O; 0–75 min 9% A; 75–100 min 20% A; λ = 240 nm; flow rate 25 ml min^{-1}; the product was collected at 86.5–90.0 min). IR: 3380*m*, 3095*m*, 2890*m*, 1670*s*, 1600*m*, 1480*w*, 1425*w*, 1200*s*, 1165*s*, 1135*s*, 835*w*, 800*m*, 720*m*. ^{1}H-NMR (D_2O): 7.12 (*d*, *J* = 3.1, 1 arom. H); 6.96 (*dd*, *J* = 3.1, 9.0, 1 arom. H); 6.85 (*d*, *J* = 9.0, 1 arom. H); 3.47 (*t*, *J* = 6.7, 2 H); 3.23–3.04 (*m*, 14 H); 2.25–1.93 (*m*, 6 H); 1.85–1.71 (*m*, 4 H). ^{13}C-NMR (D_2O): 174.0 (*s*, CONH); 167.0 (*q*, *J* = 36, 4 CO(TFA)); 155.1, 152.8 (2*s*, 2 arom. C); 126.4, 122.6 (2*d*, 2 arom. C); 121.4 (*s*, 1 arom. C); 120.6 (*q*, *J* = 291, 4 CF_3); 118.5 (*d*, 1 arom. C); 51.2, 49.5, 49.1 (3*t*); 49.0 (*t*, 2 C); 48.8, 40.9, 40.6, 30.1, 28.1 (5*t*, 5 C); 27.1 (*t*, 2 C); 27.0 (*t*). MS see paragraph 7.

5.11. N-*(16-Amino-5,9,13-triazahexadecyl)-1*H*-indole-3-acetamide* (*IndAc4333*, **AG 416b**). Treatment of **16** (1.55 mmol) according to *GP 5.1* afforded *IndAc4333*·4 HCl (97 mg, 0.173 mmol; ovrall 11% with respect to **1**) after purification by HPLC (solvent A: 0.05% HCl in MeOH; solvent B: 0.05% HCl in H_2O; 12.5% A; λ = 220 nm; flow rate 20 ml min^{-1}; the product was collected at 31.5–42.0 min). IR: 3380*s*, 3320*m*, *2950s*, 2750*s*, 2490*m*, 2410*m*, 1655*m*, 1610*w*, 1525*m*, 1455*m*, 1050*w*, 770*w*, 740*m*.^{1}H-NMR (D_2O): 7.55–7.40 (*m*, 2 arom. H); 7.25–7.03 (*m*, 3 arom. H); 3.63 (*s*, ArCH_2); 3.14–2.95 (*m*, 12

H); 2.91–2.80 (*m*, 4 H); 2.10–1.87 (*m*, 6 H); 1.53–1.33 (*m*, 4 H). ^{13}C-NMR (D_2O): 179.9 (*s*, CO); 140.9, 131.2 (2*s*, 2 arom. C); 129.6, 126.6, 124.0, 122.9, 116.6 (5*d*, 5 arom. C); 112.3 (*s*, 1 arom. C); 51.7, 49.3 (2*t*); 49.1 (*t*, 3 C); 48.6, 43.1, 41.1, 37.1, 30.1, 28.3, 27.3, 27.2, 27.1 (9*t*). MS see paragraph 7.

5.12. N-*(16-Amino-5,9,13-triazahexadecyl)-4-hydroxybenzamide* (*4-OH-Bz4333*, **AG 379a**). Treatment of **17** (1.64 mmol) according to *GP 5.1* afforded *4-OH-Bz4333*·4 HCl (250 mg, 0.48 mmol, 30% with respect to **1**) without further purification. IR: 3320*s*, 2955*s*, 2755*s*, 2480*s*, 2415*s*, 2020*w*, 1860*w*, 1635*s*, 1610*s*, 1508*s*, 1450*s*, 1270*m*, 1165*m*, 1050*s*, 850*m*, 770*s*. ^{1}H-NMR (D_2O): 7.66 (*d*, *J* = 8.8, 2 arom. H); 6.93 (*d*, *J* = 8.8, 2 arom. H); 3.37 (*t*, *J* = 6.7, 2 H); 3.26–3.05 (*m*, 14 H); 2.23–2.04 (*m*, 6 H); 1.82–1.59 (*m*, 4 H). ^{13}C-NMR (D_2O): 174.7 (*s*, CO); 163.6 (*s*, 1 arom. C); 133.8 (*d*, 2 arom. C); 129.9 (*s*, 1 arom. C); 119.9 (*d*, 2 arom. C); 52.0, 49.2, 49.13 (3*t*); 49.09 (*t*, 2 C); 48.8, 43.4, 41.0, 30.3, 28.2, 27.5 (6*t*); 27.1 (*t*, 2 C). MS see paragraph 7.

5.13. N-*(16-Amino-5,9,13-triazahexadecyl)-2,5-dihydroxybenzamide* (*2,5-(OH)$_2$-Bz4333*, **AG 395c**). Treatment of **18** (1.62 mmol) according to *GP 5.1* afforded *4-OH-Bz3334*·4 HCl (200 mg, 0.37 mmol; overall 23% with respect to **1**) without further purification. IR: 3423*s*, 2956*s*, 2756*s*, 2496*s*, 2415*s*, 1690*w*, 1650*m*, 1603*s*, 1590*s*, 1460*s*, 1265*m*, 1050*s*. ^{1}H-NMR (D_2O): 7.13 (*d*, *J* = 3.1, 1 arom. H); 6.97 (*dd*, *J* = 3.1, 8.8, 1 arom. H); 6.87 (*d*, *J* = 8.8, 1 arom. H); 3.41 (*t*, *J* = 6.5, 2 H); 3.26–3.08 (*m*, 14 H); 2.22–2.05 (*m*, 6 H); 1.84–1.63 (*m*, 4 H). ^{13}C-NMR (D_2O): 173.7 (*s*, CO); 155.0, 152.9 (2*s*, 2 arom. C); 125.9, 122.8 (2*d*, 2 arom. C); 122.0 (*s*, 1 arom. C); 118.6 (*d*, 1 arom. C); 51.9, 49.3 (2*t*); 49.1 (*t*, 3 C); 48.8, 43.3, 41.1, 30.1, 28.3, 27.5 (6*t*); 27.2 (*t*, 2 C). MS see paragraph 7.

6. *2-{1-[(*tert*-Butyl)dimethylsilyl]-1*H*-indole-3}-acetic acid*. To 1*H*-Indole-3-acetic acid (1.3 g, 7.4 mmol) in DMF (15 ml) at 0° was added NaH (0.445 g, 18.6 mmol). After 30 min at 0°, (*t*-Bu)Me_2SiCl (1.23 g, 8.1 mmol) was added. The soln. was stirred for 1 h at 0°, poured into H_2O (200 ml), and acidified with 10% aq. HCl soln. Crystallisation of the acid formed was completed by cooling the soln. to 4° for 30 min. The colorless precipitate was filtered off, washed with H_2O, and dried *in vacuo* for 15 h to give *TBSIndAcOH* (2.00 g, 6.9 mmol; 93%). M.p. (H_2O): 144–145°. IR: 3400*s*, 2929*s*, 1709*s*, 1452*s*, 1316*s*, 1258*m*, 1216*m*, 1139*s*, 970*s*, 739*s*. ^{1}H-NMR ($CDCl_3$): 10.08 (br *s*, COOH); 7.65–7.69 (*m*, 2 arom. H); 7.22–7.11 (*m*, 3 arom. H); 3.80 (*s*, CH_2); 0.94 (*s*, *t*-Bu); 0.60 (*s*, 2 Me). ^{13}C-NMR ($CDCl_3$): 177.9 (*s*, CO); 141.3, 130.5 (2*s*, 2 arom. C); 130.0, 121.7 (2*d*, 2

arom. C); 119.8 (*s*, 1 arom. C); 118.8, 114.0 (*d*, 2 arom. C); 109.7 (*s*, *C*CH_2); 31.2 (*t*, CH_2); 26.3 (*q*, *Me*$_3$C); 19.4 (*s*, Me$_3$*C*); –4.0 (*q*, Me_2Si). CI-MS: 290 (100, $[M + H]^+$).

7. *HPLC-UV(DAD)-APCI-MS and -MS/MS Investigation.* 7.1. *Equipment and Conditions.* Solvents and reagents: MeCN (HPLC grade, *Scharlau*, E-Barcelona); TFA, *purum* (*Fluka*, CH-Buchs). H_2O was purified with an *MILLI-*Q_{RG} apparatus (*Millipore*, Milford, MA, USA). Venom preparation: lyophilized venom (5.0 mg) was dissolved at 23° in TFA soln. (1% TFA in H_2O/MeCN 3:2, 60 μl). The hazy soln. was filtered through a 0.45 μm filter (*Eppendorf*, D-Hamburg), and the filter was rinsed with H_2O (20 μl). The combined filtrates provided the clear stock soln. that was stored at –20° and was used for the following HPLC-MS investigations. Samples of 1–5 μl of the venom stock soln., of 1–5 μl of a toxin stock soln. (1 mM synthetic polyamine derivatives in 0.75% TFA in H_2O/MeCN 7:3)), or of a 1:1 mixture of venom/toxin stock soln. were injected for the individual runs and were analyzed by HPLC-UV(DAD), HPLC-UV(DAD)-APCI-MS, and HPLC-UV(DAD)-APCI-MS/MS.

HPLC-UV(DAD): Instrumentation: *Waters 626-LC* system, fitted to a *996* photodiode-array detector, a *600S* controller, a *Millennium Chromatography Manager 2010* v. 2.15 (*Waters Corp.*, Milford, MA, USA), and a *Rheodyne-Rotary-7725i* rotary valve with a 5-μl loop (*Rheodyne*, Cotati, CA, USA). Column and chromatographic conditions: *Macherey-Nagel* C_{18} *HD* column (3 μm, 4.6 × 250 mm; *Macherey-Nagel*, F-Hoerdt); flow rate 0.5 ml min^{-1}. Mobile phase: step gradient over 5 min from 0 to 10 % of solvent *B*, then over 75 min from 10 to 45% of *B*, and finally over 20 min from 45 to 100% of *B* (solvent *A*: 0.1% soln. of TFA in H_2O, solvent *B*: 0.1% soln. of TFA in MeCN).

MS: APCI-MS and APCI-MS/MS experiments were performed on a *Finnigan-TSQ-700* triple-stage quadrupole instrument equipped with an atmospheric-pressure chemical-ionization (APCI) ion source (*Finnigan*, San José, CA, USA). The APCI operating conditions in positive mode were: vaporizer temp. 450°; corona voltage 5 kV; heated capillary temp. 250°; sheath gas N_2 with an inlet pressure of 40 PSI; conversion dynode: –15 kV. For MS/MS experiments: collision gas Ar with a relative pressure of 2.5–3.3 mTorr; collision-induced dissociation offset (Coff): –27 eV.

7.2. *Results of the ESI-MS/MS Investigation. See Tables 2–4.*

Table 2. *MS/MS Signals of Natural and Synthetic Compounds with Quasi-Molecular Ions* m/z *417 (*IndAc-*Pentamines).*

m/z	Signals and retention times					
	Natural venom		Synthetic samples with the *IndAc* group attached to the polyamines			
	t_R = 26.0	t_R = 27.5	t_R = 26.0 *PA3334* (**AG 416a**)	t_R = 26.0 *PA3343* (**AG 416**)	t_R = 26.0 *PA3433* (—)	t_R = 27.5 *PA4333* (**AG 416b**)
58	2	2	—	—	—	4
70	—	5	—	—	—	4
72	6	4	11	2	—	4
84	4	9	4	6	3	3
98	15	49	25	—	6	65
112	59	41	46	100	53	11
115	16	36	19	—	19	46
127	—	3	—	—	—	—
129	18	9	8	55	3	3
130	10	5	10	4	9	5
132	2	4	1	—	1	2
144	—	2	—	—	—	1
155	—	4	—	—	—	3
158	1	2	2	1	—	2
159	1	1	2	1	2	2
169	5	7	7	—	1	—
170	—	6	—	—	—	1
172	1	34	1	—	—	41
186	24	4	11	3	40	2
187	4	2	—	1	2	3
189	2	2	2	—	—	2
203	1	2	1	—	—	—
215	100	22	100	58	100	1
228	2	4	1	—	—	5
229	—	16	—	—	—	24
241	—	2	—	—	—	1
243	2	2	3	—	—	1
258	—	3	—	—	—	2
260	3	9	3	—	—	9
268	—	—	—	—	—	1
272	29	9	45	22	—	3
286	21	81	—	1	46	92
289	7	4	7	12	—	—
303	—	19	—	—	—	25
311	8	2	10	—	—	—
325	4	3	—	14	—	2
328	13	3	22	—	—	—
329	11	4	22	—	—	—
343	7	37	—	20	—	45
346	19	10	26	—	—	—
360	—	3	—	—	—	3
382	5	1	8	—	—	—
399	18	6	25	4	—	3
400	10	7	13	—	—	8
417	26	100	25	2	4	100

Table 3. *MS/MS Signals of Natural and Synthetic Compounds with Quasi-Molecular Ions* m/z *380 (*4-OH-Bz-*Pentamines).*

m/z	Signals and retention times					
	Natural venom		Synthetic samples with the 4-OH-Bz group attached to the polyamines			
	t_R = 16.7	t_R = 17.8	t_R = 16.7 PA3334 (—)	t_R = 16.7 PA3343 (**AG 379**)	t_R = 16.7 PA3433 (—)	t_R = 17.8 PA4333 (**AG 379a**)
44	1	2	—	—	—	2
58	2	5	1	2	—	3
70	3	3	—	—	—	2
72	18	3	15	3	—	2
84	9	3	5	9	3	3
98	24	82	29	—	8	100
112	81	33	48	100	51	16
115	22	54	17	—	18	49
121	14	18	11	5	12	15
129	25	7	12	46	1	5
132	3	4	1	—	2	4
138	—	4	—	—	—	—
148	—	—	1	—	—	—
150	2	3	1	—	—	—
155	—	4	—	—	—	2
169	8	3	7	—	—	—
172	3	34	3	—	—	39
176	—	2	—	—	—	—
178	100	10	100	48	100	1
186	9	4	8	1	18	2
189	1	2	4	—	—	
191	5	3	2	2	—	
192	—	42	—	—	—	34
203	2	—	—	—	—	—
205	—	2	—	—	—	—
206	—	—	1	—	—	—
207	2	—	2	—	—	—
220	—	—	2	—	—	—
221	2	2	—	—	—	—
235	49	10	34	18	—	2
243	3	5	9	—	—	2
249	3	100	—	—	25	85
252	10	2	11	5	—	—
260	5	31	9	—	—	22
266	—	12	—	—	—	16
274	7	—	7	—	—	—
288	4	2	—	4	—	2
292	24	3	19	—	—	—
306	5	43	—	11	—	38
309	23	3	11	—	—	—
322	—	—	—	—	—	3
323	—	3	—	—	—	—
345	2	—	4	—	—	—
362	11	5	10	1	—	3
363	5	5	9	—	—	5

Table 4. *MS/MS Signals of Natural and Synthetic Compounds with Quasi-Molecular Ions* m/z *396 (*2,5-$(OH)_2$-Bz-*Pentamines).*

m/z	Signals and retention times					
	Natural venom		Synthetic samples with the 2,5-$(OH)_2$-Bz group attached to the polyamines			
	t_R = 19.5	t_R = 21.8	t_R = 19.5 PA3334 (**AG 395b**)	t_R = 19.5 PA3343 (—)	t_R = 19.5 PA3433 (—)	t_R = 21.8 PA4333 (**AG 395c**)
44	3	8	—	—	—	
58	2	6	2	3	1	3
70	6	6	2	—	—	4
72	25	—	27	2	—	3
84	5	7	6	7	3	3
98	39	91	44	—	9	100
112	100	29	69	100	55	15
115	28	47	33	3	22	65
129	36	43	15	50	10	21
132	5	—	3	—	2	5
137	9	11	13	5	9	5
141	—	—	1	—	—	—
146	—	—	—	—	—	2
155	—	11	—	—	—	3
166	4	—	2	1	4	—
169	11	—	8	—	—	2
172	15	58	9	—	—	37
186	16	29	10	10	22	12
189	12	7	15	—	—	5
194	91	15	100	23	100	—
203	2	—	2	—	—	—
207	3	8	2	—	—	4
208	—	24	—	—	—	14
223	3	—	1	—	—	—
237	—	12	—	—	—	2
243	13	16	15	—	—	4
251	82	10	49	19	—	2
260	10	70	18	—	1	42
265	3	66	—	—	38	70
268	11	—	5	4	—	—
282	—	22	—	—	—	12
290	7	—	10	—	—	—
304	3	7	—	6	—	—
308	46	10	28	—	—	—
322	8	100	—	17	—	55
325	27	14	29	—	—	—
338	—	—	—	—	—	—
339	—	9	—	—	—	3
361	6	—	4	—	—	—
378	21	—	11	—	—	1
379	21	12	10	—	—	5
396	30	32	18	—	2	61

References

[1] A. Schäfer, H. Benz, W. Fiedler, A. Guggisberg, S. Bienz, M. Hesse, in 'The Alkaloids', Ed. G. A. Cordell and A. Brossi, Academic Press, New York, 1994, Vol. 45, p. 1.

[2] A. L. Mueller, R. Roeloffs, H. Jackson, in 'The Alkaloids', Ed. G. A. Cordell and A. Brossi, Academic Press, San Diego, CA, 1995, Vol. 46, p. 63.

[3] G. Karigiannis, D. Papaioannou, *Eur. J. Org. Chem.* **2000**, 1841.

[4] S. Bienz, R. Detterbeck, C. Ensch, A. Guggisberg, U. Häusermann, C. Meisterhans, B. Wendt, C. Werner, M. Hesse, in 'The Alkaloids', Ed. G. A. Cordell, Academic Press, New York, 2002, p. 83.

[5] S. Chesnov, L. Bigler, M. Hesse, *Helv. Chim. Acta* **2001**, *84*, 2178.

[6] S. Chesnov, L. Bigler, M. Hesse, *Helv. Chim. Acta* **2000**, *83*, 3295.

[7] N. A. Saccomano, R. A. Volkmann, Eur. Pat. Appl. 436332, 1990; *Chem. Abstr.*, **1990**, *115*, 231996p.

[8] B. G. Quistad, C. C. Reuter, W. S. Skinner, P. A. Dennis, S. Suwanrumpha, E. W. Fu, *Toxicon* **1991**, *29*, 329.

[9] N. Manov, S. Bienz, *Tetrahedron* **2001**, *57*, 7893.

[10] P. Conti, D. Demont, J. Cals, H. C. J. Ottenheijm, D. Leysen, *Tetrahedron Lett.* **1997**, *38*, 2915.

[11] U. Kramer, A. Guggisberg, M. Hesse, H. Schmid, *Helv. Chim. Acta* **1977**, *62*, 811.

[12] E. J. Corey, D. J. Brunelle, K. C. Nicolaou, *J. Am. Chem. Soc.* **1977**, *99*, 7359.

[13] H. Stach, M. Hesse, *Helv. Chim. Acta* **1886**, *69*, 85.

[14] S. Bienz, A. Guggisberg, M. Hesse, *Helv. Chim. Acta* **1988**, *71*, 1708.

[15] L. Bigler, C. F. Schnider, W. Hu, M. Hesse, *Helv. Chim. Acta* **1996**, *79*, 2152.

[16] K. Samejima, Y. Takeda, M. Kawase, M. Okada, Y. Kyogoku, *Chemical & Pharmaceutical Bulletin* **1984**, *32*, 3428.

[17] V. K. Sarin, S. B. Kent, J. P. Tam, R. B. Merrifield, *Anal. Biochem.* **1981**, *117*, 147.

CHAPTER 2

Tandem Mass Spectrometric Investigation of Acylpolyamines of Spider Venoms and their ^{15}N-Labeled Derivatives[1]

Abstract

The fragmentation mechanism of the acylpentamine toxins **1–4** found in the venom of the spider *Agelenopsis aperta* has been investigated in detail. To identify the origin of the two doublets of unexpected fragment ions at *m*/*z* 129/112 and *m*/*z* 115/98, three synthetic ^{15}N-labeled analogs **5–7** have been prepared and subjected to CID fragmentation on a triple quadrupole mass spectrometer. It appears that the unexpected doublet of fragment ions arises from an internal portion of the polyamine backbone after either a transaminative *Zip*-reaction or a sequential fragmentation of the quasi-molecular ion. The second option has been proven by in-source CID experiments. The detailed knowledge of acylpentamine fragmentation mechanisms is essential for the correct characterization of isomeric compounds, particularly for co-eluting compounds within complex mixtures such as spider venoms.

Introduction

Polyamines and polyamine-containing alkaloids are widely found throughout the plant and animal kingdom [1, 2]. Over the past decades, spider venoms, particularly their fractions containing acylpolyamine derivatives [1, 3, 4], have increasingly attracted the attention of scientists. With the advent of more sensitive and selective analytical methods — in particular of the modern mass spectrometric approaches — not only the major but also minor constituents of such trace toxin sources became amenable to detection and structural elucidation. Only recently have we demonstrated with the reinvestigation of the venom of the spider *Agelenopsis aperta* (Agelenidae) that this toxin cocktail contains in addition to the previously found 11

[1] M. Tzouros, N. Manov, S. Bienz, L. Bigler, *J. Am. Soc. Mass Spectrom.* **2004**, *15*, 1636.

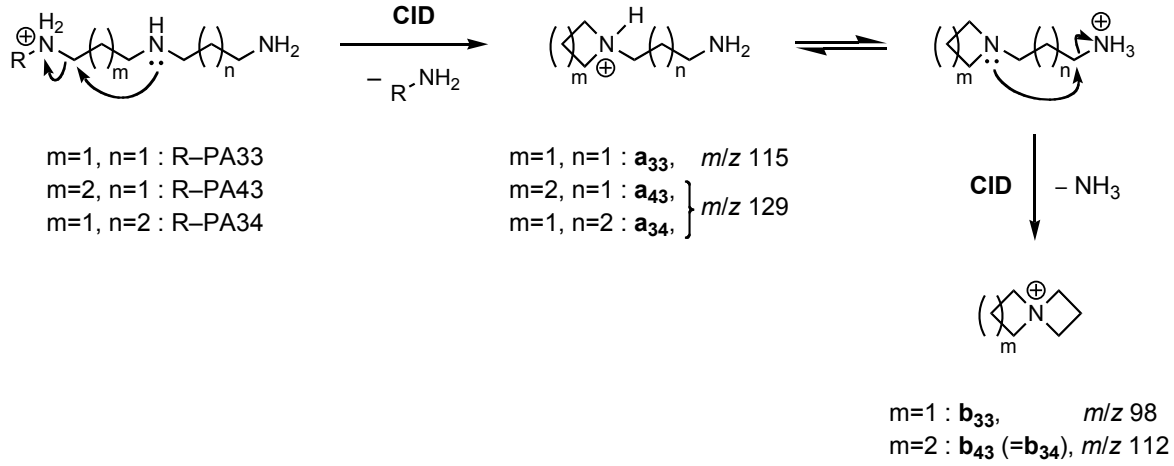
major acylpolyamines, 27 further minor components that evaded earlier investigations [5, 6].

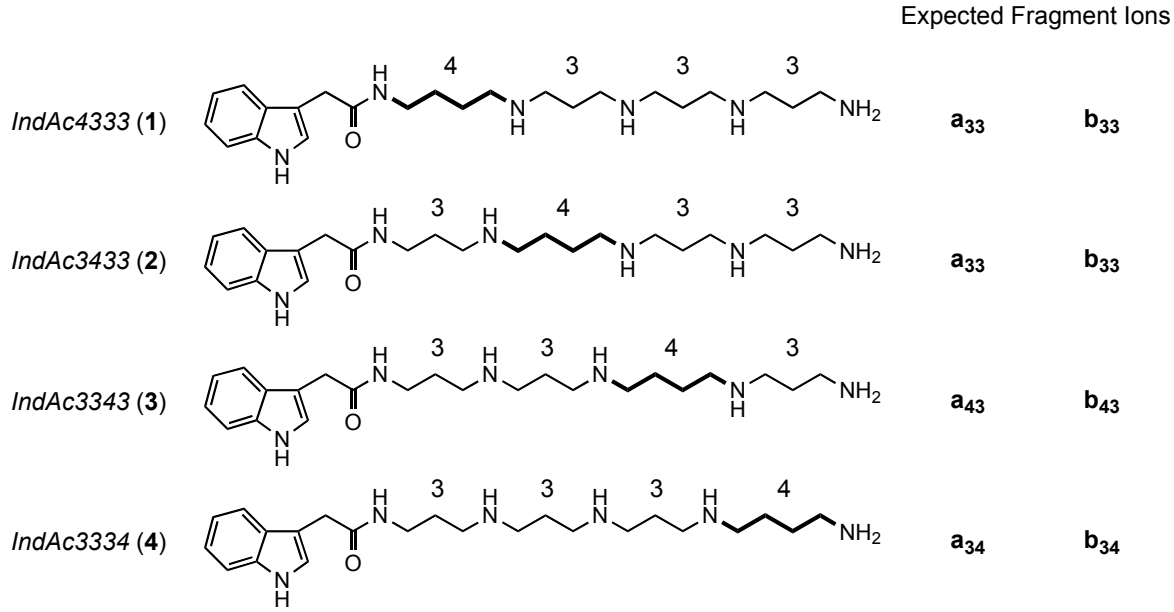
The detection and structural elucidation of these spider toxins relied particularly on the on-line coupled high-performance liquid chromatography, UV diode array detection, and atmospheric-pressure chemical ionization mass spectrometry (HPLC-UV(DAD)-APCI-MS and -MS/MS). This arrangement allowed the efficient separation and investigation of the several venom components, making most economical use of the precious sample material. The structural elucidations, however, would not have been possible if a profound knowledge of the fragmentation behavior of the parent class of compounds had not been available, especially in the case of co-eluting isomers. While most of the toxins of *A. aperta* were disclosed on the basis of pure 'MS investigations' by comparison of fragmentation patterns observed and explained during previous investigations, five of them were only revealed by their comparison with synthetic material [6]. But also in these cases, their identity could only be revealed because of the specific fragmentation behavior of several structural isomers that could only be studied, however, with the pure sample compounds. On the whole, the knowledge of the fragmentation patterns of polyamines and also the deeper understanding of the respective fragmentation mechanisms proved an important prerequisite to allow efficient and definitive identification and characterization of known and new polyamine derivatives by MS methods.

In the course of our investigation of the venom of *A. aperta*, we have studied the fragmentation behavior of acylpolyamines extensively and have also discussed possible fragmentation paths and mechanisms [5]. For some groups of isomeric acylpentamines, however, inconsistencies with regard to expected fragmentation patterns surfaced. Concentrating solely on the proposed fragmentation reaction for the 'loss of the terminal diaminopolymethylene unit', the occurrence of fragments of the types **a** and **b** — formed upon collision induced dissociation (CID) by intramolecular nucleophilic substitutions (s_Ni) according to *Scheme 1* — were thought to be indicative for the identification of the terminal diamine portions of the toxins. However, this is only true with reservation. For instance, the toxins *IndAc4333*[2] (**1**)

[2]The expression *IndAc4333* stands for a linear pentamine possessing 4, 3, 3, and 3 methylene units between the several N-atoms, whereby the *IndAc* group is located at the N-atom which is followed by the tetramethylene unit. *IndAc* is the abbreviation for 1*H*-indole-3-acetyl.

and *IndAc3433* (**2**) would be expected to give rise to fragments $\mathbf{a}_{33}$[3] (*m*/*z* 115) and $\mathbf{b}_{33}$ (*m*/*z* 98) and not to fragments $\mathbf{a}_{43}$/$\mathbf{a}_{34}$ (*m*/*z* 129) and $\mathbf{b}_{43}$ (*m*/*z* 112), while isomeric *IndAc3343* (**3**) and *IndAc3334* (**4**) should produce fragments $\mathbf{a}_{43}$/$\mathbf{a}_{34}$ and $\mathbf{b}_{43}$ instead, and none of the fragments $\mathbf{a}_{33}$ and $\mathbf{b}_{33}$.





Scheme 1. *Mass spectral fragmentation of the terminal part of the [M + H]⁺ ions of the* IndAc *pentamine derivatives* ***1***–***4***.

[3] The subscripts denote the number of methylene groups contained in the two chains or rings.

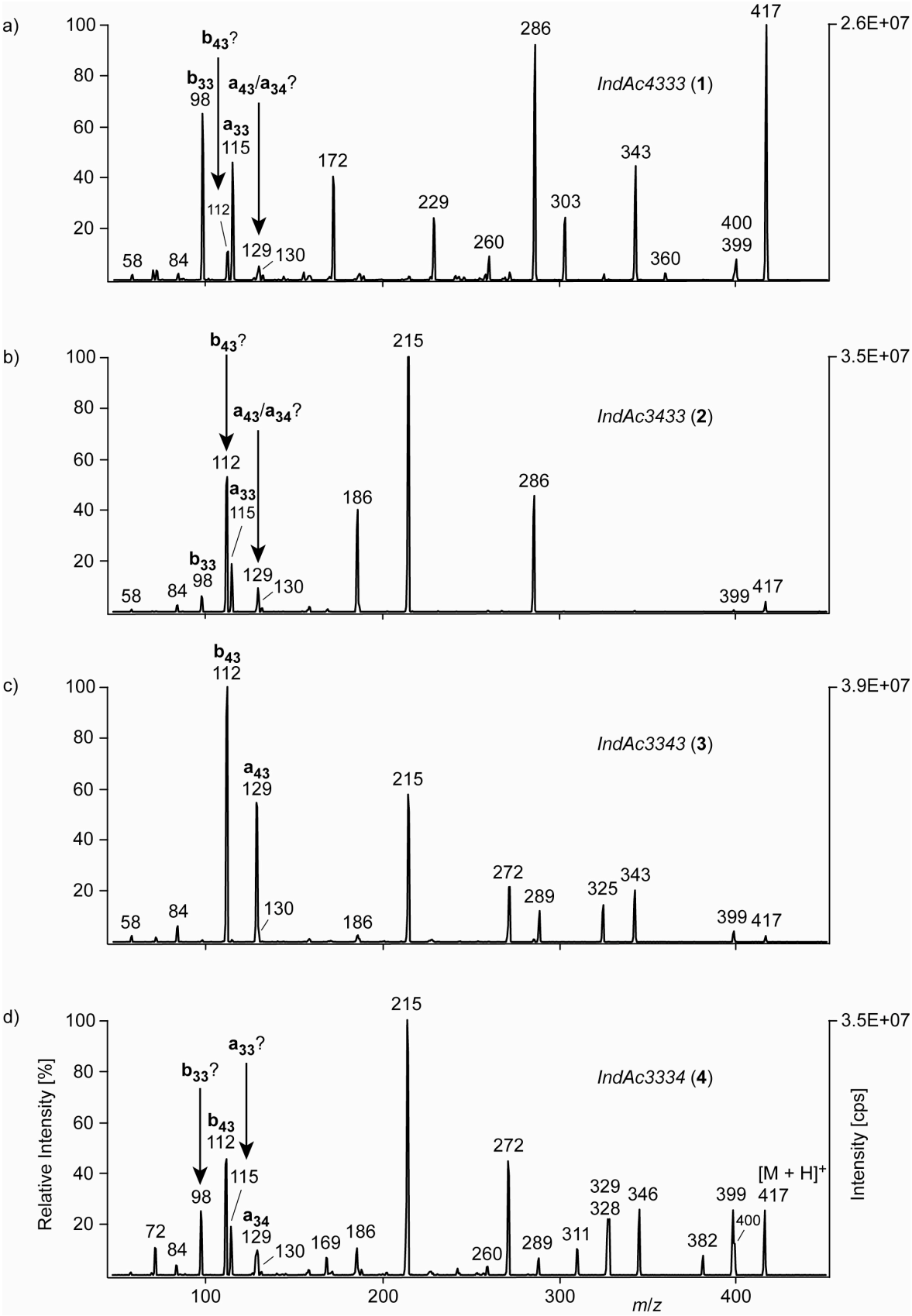
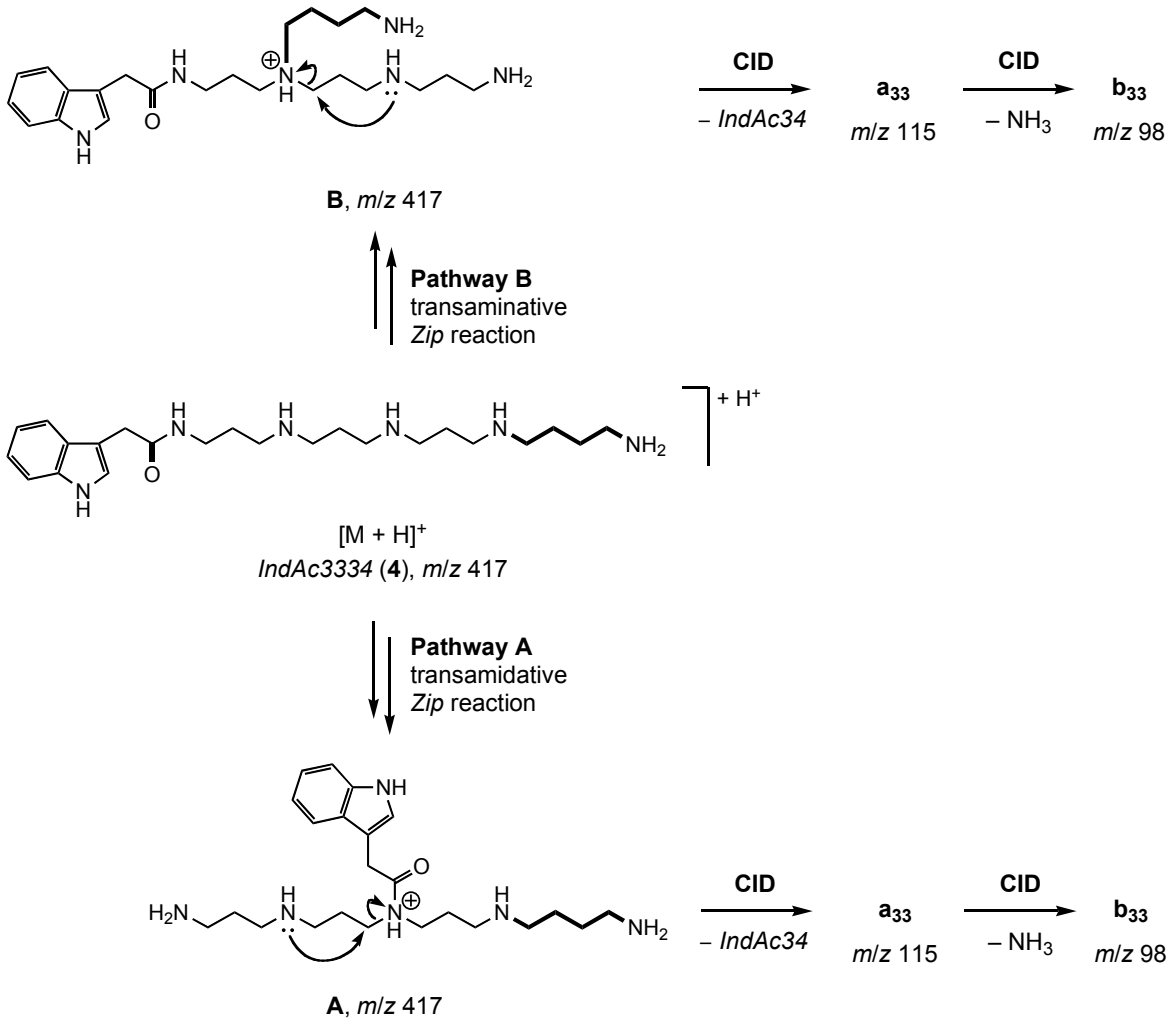


Figure 1. *ESI-MS/MS of the [M + H]⁺ ions at* m/z *417 of a)* IndAc4333 *(**1**), b)* IndAc3433 *(**2**), c)* IndAc3343 *(**3**), and d)* IndAc3334 *(**4**).*
Unexpected fragment ions are indicated by an arrow.

In reality, however, the CID spectra deriving from the two quasi-molecular ions of synthetic *IndAc4333* and *IndAc3433* also showed signals at *m*/*z* 129 and 112, which should be indicative for a terminal *PA34* or *PA43* unit[4], and in the case of *IndAc3334* (but not of *IndAc3343*!), signals at *m*/*z* 115 and 98 were additionally found, which would imply a *PA33* end-portion for the molecule (*Figure 1*). The feature of exhibiting unexpected signals of such types is not specific to pentamines bearing the *IndAc* moiety; it is also characteristic for other pentamine derivatives that bear, *e.g.*, a 4-hydroxybenzoic or a 2,5-dihydroxybenzoic group in place of *IndAc* (spectra not shown here).



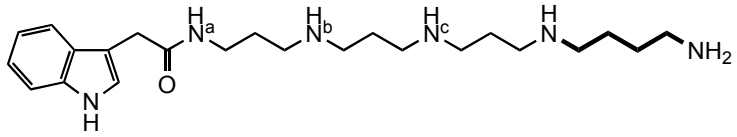
Scheme 2. *Possible gas phase rearrangements of the [M + H]⁺ ions of* IndAc3334 *(**4**).*

As an explanation for the peculiar MS behavior of the acylpentamines, we have offered two plausible reaction paths involving an acid promoted transamidative (Pathway A) or transaminative (Pathway B) *Zip*-reaction [7], as outlined in *Scheme 2*

[4]PA34 stands for a linear triamine with 3 and 4 methylene units (in this succession) between the terminal and internal N-atoms [2]. The nomenclature is also used for more extended polyamines.

in the case of *IndAc3334* (**4**). For both transformations, precedence is available in the MS literature: transamidation reactions were discussed with respect to the MS behavior of 4-hydroxycinnamoyl spermidines [8] as well as *N*,*N*′-bis(4-hydroxycinnamoyl) spermidines [9], and transamination reactions were considered for polypropylenamine dendrimers [10] and for monoimidazole polyamine conjugates [11]. The presence of the signal doublet at *m*/*z* 129/112 in the CID of *IndAc4333* would rather support the transamidative Pathway A while the lack of signals at *m*/*z* 115/98 for *IndAc3343* would suggest Pathway B proceeding through transaminations.

To distinguish between these two paths, we have synthesized the three ^{15}N-containing *IndAc3334* derivatives *IndAc*^{15}*N3334* (**5**), *IndAc3*^{15}*N334* (**6**), and *IndAc33*^{15}*N34* (**7**) [12] (*Figure 2*). The three labeled isomers were then analyzed by ESI-MS/MS, analogously to the unlabeled parent compound. The results of this MS investigation are presented below.



N^a = ^{15}N : *IndAc*^{15}*N3334* (**5**), $[M + H]^+$ *m*/*z* 418
N^b = ^{15}N : *IndAc3*^{15}*N334* (**6**)
N^c = ^{15}N : *IndAc33*^{15}*N34* (**7**)

Figure 2. *Investigated* ^{15}*N-labeled* IndAc3334 *derivatives* **5**–**7**.

Experimental Part

Material

All ^{15}N-labeled acylpolyamine samples used were synthesized in our laboratory [12]. For ESI-MS, all compounds were dissolved in a 1:1 mixture of HPLC grade MeOH (Scharlau, Barcelona, Spain) and H_2O (purified with a Milli-Q_{RG} apparatus, Millipore, Milford, MA) at a concentration of 50 nmol ml^{-1}.

Mass Spectrometry

ESI tandem mass spectra were recorded with a TSQ 700 triple quadrupole mass spectrometer (Finnigan MAT, San Jose, CA) equipped with a combined Atmospheric

Pressure Ion (API) source. Samples were continuously introduced into the source with a Syringe Pump 22 (Harvard Apparatus, Holliston, MA) at a flow rate of 5 μl min^{-1}. The ESI operating conditions in positive mode were: capillary voltage 4500 V; heated capillary temperature 210°C; sheath gas N_2 with an inlet pressure of 30 psi; electron multiplier: 1000 V; conversion dynode: –15 kV; resolution: 0.7 u at half peak height; scan rate: 900 Da s^{-1}; 16 scans were averaged. For the CID experiments, Ar was used as the collision gas with a relative pressure of 2.5–3.3 mTorr. The collision-induced dissociation offset (Coff) was set to –27 V and the electron multiplier was set to 1500 V. In-source CID spectrum was obtained by setting the octopole voltage at –3 V, and fragment ions were further subjected to CID by setting the Coff voltage at –22 V.

Results and Discussion

The ESI-MS/MS of the three labeled compounds **5–7** and of the unlabeled parent compound **4** — all obtained under the same conditions from the respective $[M + H]^+$ parent ions — are shown in *Figure 3*. Concentrating on the signals of type **a** and **b**, it is readily recognized that the signals for the respective fragment ions $\mathbf{a_{34}}/\mathbf{b_{43}}$[5] at *m*/*z* 129/112 remained unaffected for all the ^{15}N-containing compounds. However, the signals for the $\mathbf{a_{33}}/\mathbf{b_{33}}$ ions were recorded only for *IndAc*^{15}*N3334* (**5**) and *IndAc3*^{15}*N334* (**6**) at *m*/*z* 115/98 while they were found at masses higher by 1 Da (*m*/*z* 116/99) for *IndAc33*^{15}*N34* (**7**) and consequently designated as ^{15}N-$\mathbf{a_{33}}$ and ^{15}N-$\mathbf{b_{33}}$, respectively.

These results unambigously show that the central portion of the polyamine moiety (as indicated by the surrounded part of the compound in *Figure 4*) is incorporated in the fragments $\mathbf{a_{33}}/\mathbf{b_{33}}$ deriving from compound **4.** Therefore, the transamidative Pathway A for the formation of these fragments is certainly not operative and can be excluded. Otherwise, doublets for labeled fragments $\mathbf{a_{33}}/\mathbf{b_{33}}$ at *m*/*z* 116/98 or 116/99 should have been found for isomers **5** and **6**, respectively. On the other hand, the doublet of signals at *m*/*z* 116/99 for the ^{15}N-containing fragments $\mathbf{a_{33}}/\mathbf{b_{33}}$ deriving from isomer **7** is supportive for the transaminative Pathway B.

[5] The fragment ion at *m*/*z* 130 has been previously described as 3-methylene-3*H*-indolium ($C_9H_8N^+$) [5] and is not considered as labeled ^{15}N-$\mathbf{a_{43}}/\mathbf{a_{34}}$.

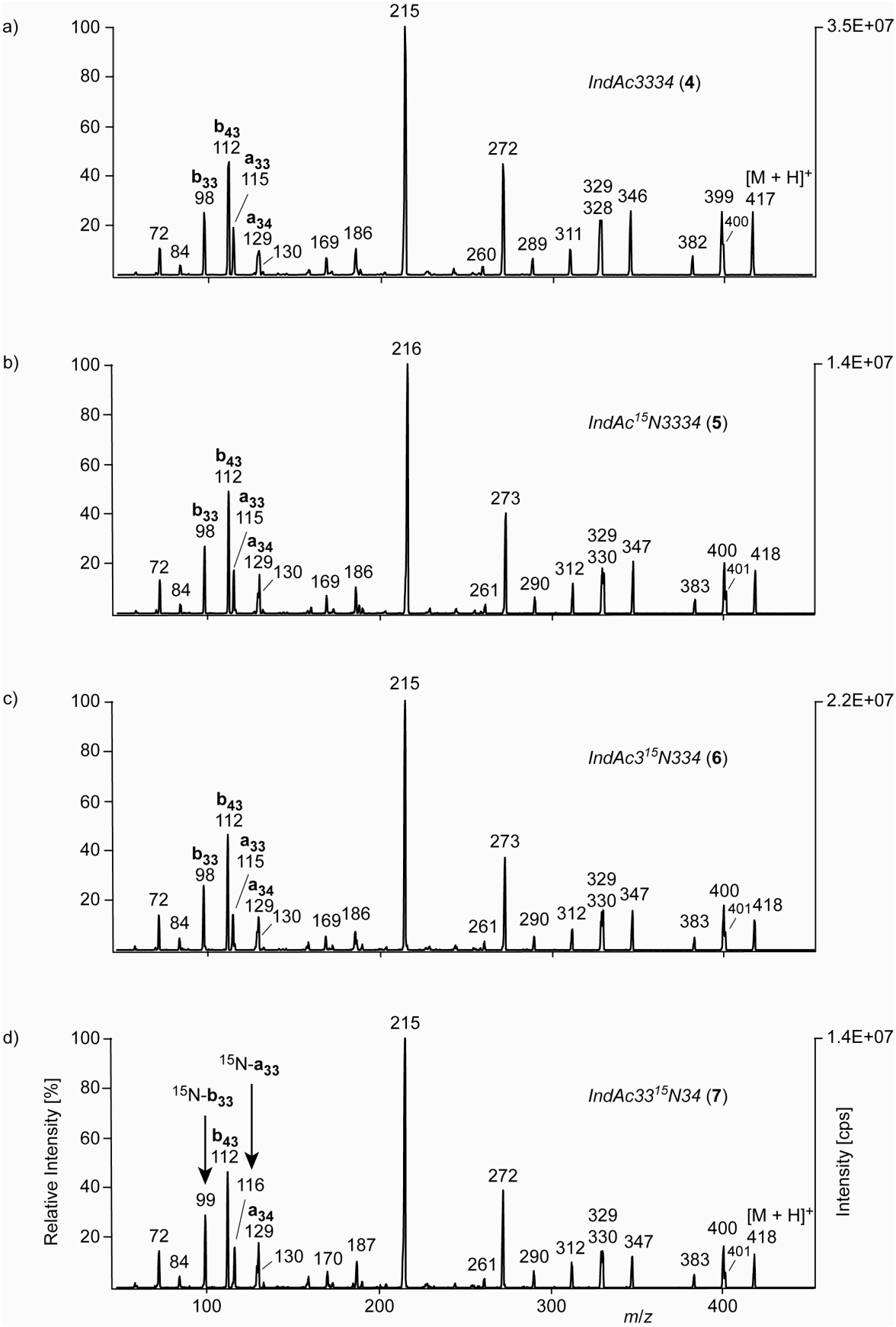


Figure 3. *ESI-MS/MS of the [M + H]⁺ ions at* m/z *417 of a)* IndAc3334 (***4***)*, and at* m/z *418 of labeled b)* IndAc^{15}N3334 (***5***)*, c)* IndAc3^{15}N334 (***6***)*, and d)* IndAc33^{15}N34 (***7***)*.* ^{15}*N-label containing* ***a*** *and* ***b*** *fragment ions are indicated by an arrow.*

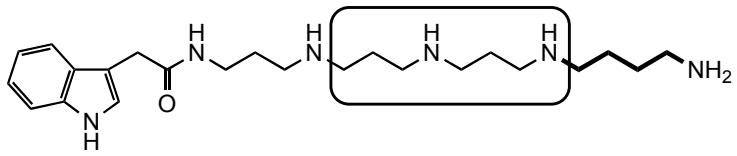
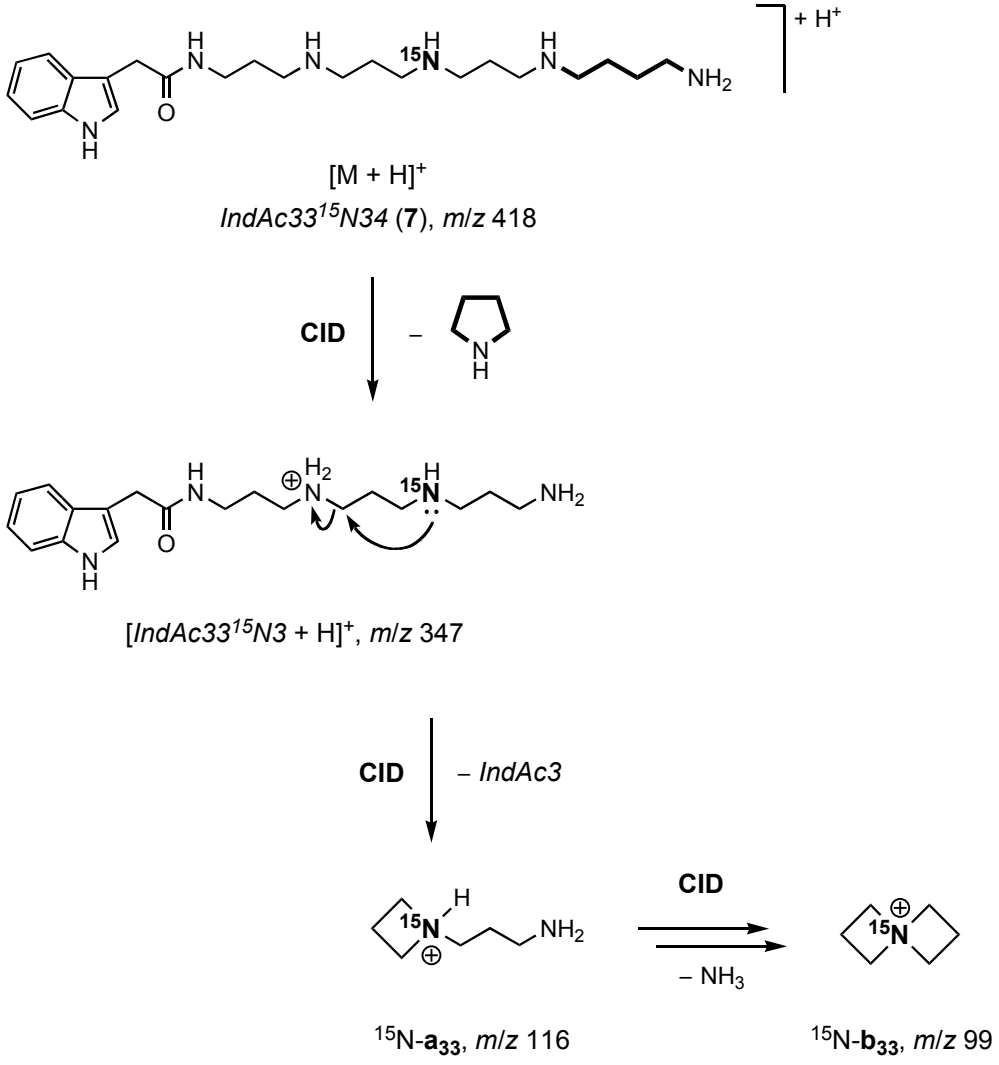


Figure 4. *Portion of the polyamine backbone of* IndAc3334 *responsible for the formation of the fragment ions* $\boldsymbol{a_{33}}$/$\boldsymbol{b_{33}}$ *at* m/z *115/98.*

An alternative fragmentation path that was not seriously considered initially, but is also consistent with the experimental data, is the sequential fragmentation of acylpolyamines involving loss of a terminal aminoalkyl group in a first stage, forming, *e.g.*, for *IndAc3334* the ion [*IndAc333* + H]$^+$. Such a secondary parent ion could subsequently be fragmented according to the general rules (*Scheme 3*). This would lead to fragments $\mathbf{a_{33}}$ and $\mathbf{b_{33}}$ that would consist of the same portions of the parent molecule as those explained with the transamination/fragmentation pathway.



Scheme 3. *Sequential mass spectral fragmentation of the [M + H]$^+$ ions of* IndAc33^{15}N34 *(**7**).*

However, if *IndAc3334* dissociates by the usual s_Ni reaction by attack of the terminal amino group at the CH_2 group in α-position to the protonated secondary N-atom, protonated pyrrolidine (see fragment ions at *m*/*z* 72 in *Figure 3*) and neutral *IndAc333* is formed. Thus the observed $[IndAc33^{15}N3 + H]^+$ at *m*/*z* 347 from $IndAc33^{15}N34$ (**7**) cannot directly derive from this s_Ni reaction. In order to retain the charge on the *IndAc*-end, a proton transfer process has to be operative. It is possible that a proton-bound complex is formed during fragmentation, where the *IndAc333*-unit and pyrrolidine compete for the H^+. This mechanism was postulated to explain the formation of the b_n/y_n ion series in the case of peptide fragmentation [13]. Support for the sequential fragmentation of **4**–**7** is found with the signals at *m*/*z* 346 and 347, corresponding to the proposed ions $[IndAc333 + H]^+$ (*Figure 3*). That these ions are formed rather easily is also demonstrated with the in-source CID experiment, which involves very mild energy transfer and thus very mild fragmentation conditions, performed with $IndAc33^{15}N34$ (**7**) (*Figure 5*). By in-source CID, almost exclusively the respective ions $[IndAc33^{15}N3 + H]^+$ registered at *m*/*z* 347 were formed. These ions, selected by the first quadrupole and subjected to the usual CID by collision with Ar in the collision cell, gave rise to a spectrum that exhibited, not surprisingly, signals at *m*/*z* 116 and 99 for the ^{15}N-labeled fragments of type $\mathbf{a_{33}}$ and $\mathbf{b_{33}}$.

Based on the experimental data acquired by the above experiments, both mechanisms — dissociation of the quasi-molecular ion $[IndAc3334 + H]^+$ through transamination/fragmentation (Pathway B) or by sequential fragmentation *via* intermediary $[IndAc333 + H]^+$ ions — are feasible to explain to the formation of fragments $\mathbf{a_{34}}/\mathbf{b_{34}}$ detected at *m*/*z* 129/115 in the ESI-MS/MS of compound **4**. The fact that $[IndAc333 + H]^+$ is formed quite easily by in-source CID and that this ion is also detected in the regular CID spectra talks in favor of the sequential fragmentation path.

The behaviors of *IndAc3433* (**2**) and *IndAc3343* (**3**) are consistent with the two fragentation paths discussed above as well. In the case of *IndAc3433* (**2**), both loss of azetidine or transamination would form intermediates that could give rise to fragments $\mathbf{a_{43}}$ and $\mathbf{b_{43}}$ and thus to the observed signals at *m*/*z* 129/112 (see *Figure 1*b). In this case, the signal for the secondary parent ion $[IndAc343 + H]^+$ (*m*/*z* 360) proposed for the stepwise pathway is not found in the spectrum, which is possibly due to the fact that this ion should dissociate immediately after its formation to

fragment $\mathbf{a_{43}}$ — and subsequently $\mathbf{b_{43}}$ — through a favored five-membered transition structure as described above. For *IndAc3343* (**3**), the doublet at *m*/*z* 129/112 can be the response to the fragments $\mathbf{a_{43}}/\mathbf{a_{34}}$ and $\mathbf{b_{34}}$ deriving either by direct substitutive fragmentation or by the more complex pathways discussed above (see *Figure 1*c). A doublet at *m*/*z* 115/98 for $\mathbf{a_{33}}$ and $\mathbf{b_{33}}$ is not observed for this compound, however, because [*IndAc33* + H]$^+$ (*m*/*z* 289), obtained by loss of terminal diaminoalkyl group, cannot undergo fragmentation to these ions similarly to a free triamine.

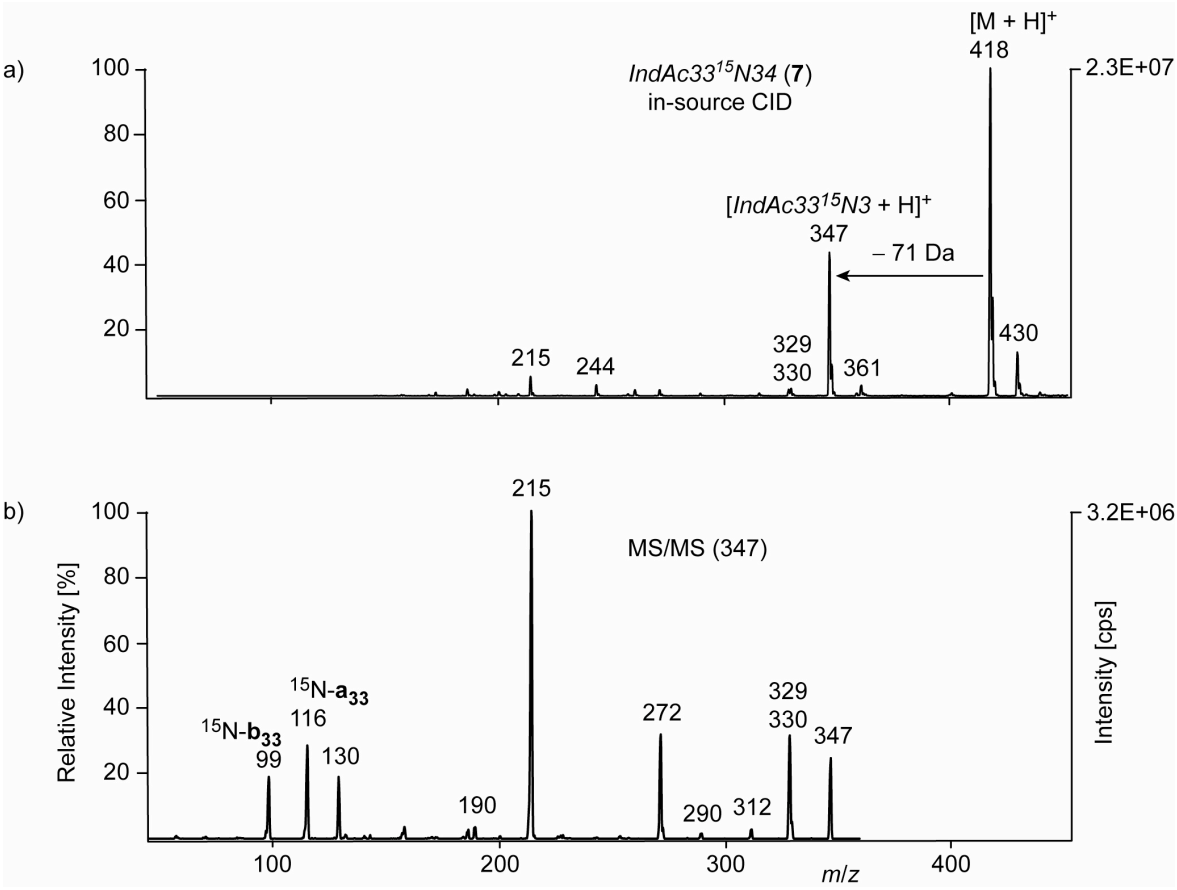


Figure 5. *In-source CID of the [M + H]$^+$ ions at* m/z *418 of labeled a)* IndAc33^{15}N34 *(**7**), and b) MS/MS of the secondary parent [*IndAc33^{15}N3 + *H]$^+$ ions at* m/z *347.*

In the case of *IndAc4333* (**1**), the explanation for the formation of the ions of type $\mathbf{a_{43}}/\mathbf{a_{34}}$ and $\mathbf{b_{43}}$ (see *Figure 1*a) is slightly different: loss of any portion of the molecule from the amino terminus of the compound and also transaminations would never lead to an intermediary species that could form these fragments. However, loss of the acyl group and liberation of the protonated polyamine itself seems to be a favorable CID-reaction for *Acyl4* derivatives [6]. The respective signal for [*PA4333* + H]$^+$ is in fact found at *m*/*z* 260 in the ESI-MS/MS of *IndAc4333* (**1**), and decomposition of the free polyamine can give rise to the fragments $\mathbf{a_{43}}/\mathbf{a_{34}}$ and $\mathbf{b_{43}}$. The corresponding

$[PA4333 + H]^+$ and $[PA3433 + H]^+$ signals for compounds **2–4** are of low abundance only or not observed at all (see *Figure 1*a-d). For *Acyl3* derivatives, the alternative formation of 4*H*-5,6-dihydro-1,3-oxazine species, represented in the spectra by the strong signals at *m*/*z* 215, is the dominant reaction of the head portion [6].

Conclusion

We have shown that acylpolyamines can form some fragments in ESI-MS/MS that are composed of internal portions of the polyamine moieties of the sample molecules. From a mechanistic point of view, a fragmentation path involving a transamidation was excluded. Two alternative mechanisms — by transamination followed by fragmentation, or by two subsequent fragmentations — are possible and cannot be distinguished. Signals of such fragments could lead to misinterpretations if they were regarded to be derived from the end-portion of the compounds. Thus, for the definite characterization and structural identification of acylpolyamines, particularly those natural products of low abundance that lack synthetic references, such fragments are of little relevance. Of more relevance is the overall fragmentation pattern of the samples, which is dominated by the presence and location of 1,4-diaminobutane subunits. Such subunits allow some specific and predominant fragmentation reactions, occurring *via* five-membered transition structures. To deduce a specific sample structure on the basis of MS behavior, it is consequently necessary to judge the overall behavior of the considered compounds. In certain cases, the structural deduction might even not be feasible directly without the possibility to compare the sample compound with authentic samples.

References

[1] A. Schäfer, H. Benz, W. Fiedler, A. Guggisberg, S. Bienz, M. Hesse, *Alkaloids* **1994**, *45*, 1.

[2] S. Bienz, R. Detterbeck, C. Ensch, A. Guggisberg, U. Häusermann, C. Meisterhans, B. Wendt, C. Werner, M. Hesse, *Alkaloids* **2002**, *58*, 83.

[3] Y. Itagaki, T. Nakajima, *J. Toxicol. Toxin Reviews* **2000**, *19*, 23.

[4] S. Chesnov, L. Bigler, M. Hesse, *Eur. J. Mass Spectrom.* **2002**, *8*, 1.

[5] S. Chesnov, L. Bigler, M. Hesse, *Helv. Chim. Acta* **2001**, *84*, 2178.

[6] N. Manov, M. Tzouros, S. Chesnov, L. Bigler, S. Bienz, *Helv. Chim. Acta* **2002**, *85*, 2827.

[7] U. Kramer, A. Guggisberg, M. Hesse, H. Schmid, *Angew. Chem.* **1977**, *89*, 899.

[8] W. Hu, E. Reder, M. Hesse, *Helv. Chim. Acta* **1996**, *79*, 2137.

[9] L. Bigler, C. F. Schnider, W. Hu, M. Hesse, *Helv. Chim. Acta* **1996**, *79*, 2152.

[10] J. De Maaijer-Gielbert, C. Gu, A. Somogyi, V. H. Wysocki, P. G. Kistemaker, T. L. Weeding, *J. Am. Soc. Mass Spectrom.* **1999**, *10*, 414.

[11] C. Zhu, Y. Jiang, X. Yang, Y. Zhao, *Rapid Commun. Mass Spectrom.* **2002**, *16*, 2273.

[12] N. Manov, M. Tzouros, L. Bigler, S. Bienz, *Tetrahedron* **2004**, *60*, 2387.

[13] M. J. Polce, D. Ren, C. Wesdemiotis, *J. Mass Spectrom.* **2000**, *35*, 1391.

CHAPTER 3

New Linear Polyamine Derivatives in Spider Venoms[1]

Abstract

Linear free polyamines were characterized in the venom of the spiders *Agelenopsis aperta*, *Hololena curta*, and *Paracoelotes birulai* by RP-HPLC coupled to mass spectrometry. The several linear polyamines found were tetramine, pentamine, and hexamine derivatives. Some of these natural products were identified as N-hydroxylated, guanidylated, or acetylated compounds. In addition, the biosynthetical pathway leading to the formation of acylpolyamines in spider venoms is discussed.

Introduction

Spider venoms are known to be the source of a multitude of natural products and particularly of acylpolyamines [1-3]. Although many acylpolyamines with rather uncommon polyamine backbones were found in spider venoms, information about the presence of the respective free non-derivatized bases is scarce in literature. Low molecular weight biogenic amines such as the ubiquitous putrescine (*PA4*)[2], cadaverine (*PA5*), spermidine (*PA34*), and spermine (*PA343*) were characterized in the mygalomorph spiders *Scodra griseipes* [5, 6], the Sydney funnel-web spider *Atrax robustus* [7], and the species *Dugesiella hentzi*, *Aphonopelma emilia* and two further *Aphonopelma* species [8]. The same biogenic amines along with histamine were also reported in nine typical Japanese spider species [9]. Not in the venom but in the silk glands and in the heads of the spiders *Nephila clavata* and *Araneus ventricosus*, caldopentamine (*PA3333*) was found in addition to the more common biogenic amines [10]. Apart from this last example, no information about spider derived, non-acylated polyamines containing more than four N-atoms is found in literature. The only natural sources for such extended polyamines, namely pentamines and

[1] M. Tzouros, S. Chesnov, S. Bienz, M. Hesse, L. Bigler, *Toxicon* **2005**, *46*, 0000.
[2] The abbreviation *PA* stands for polyamine; the figures designate the number of methylene in-between the several N-atoms [4].

hexamines, are bacteria. For example, homocaldopentamine (*PA3334*) [11] and isomeric thermopentamine (*PA3343*) [12] were extracted from the thermophilic eubacteria *Thermus thermophilus* and homocaldohexamine (*PA33334*), thermohexamine (*PA33343*), and homothermohexamine (*PA33433*) in *Bacillus schlegelii* [13]. In these cases, also branched polyamines were characterized in addition to the linear compounds. These polyamines from bacteria were identified either by gas chromatography coupled to mass spectrometry (GC-MS) after their derivatization with heptafluorobutyric anhydride (HFBA) [14], or by liquid chromatography (LC) and fluorescence detection of the *o*-phthalaldehyde (OPA) reaction products [15].

Recently, we have used high-performance liquid chromatography, on-line coupled with atmospheric-pressure chemical ionization mass spectrometry (HPLC-UV(DAD)-APCI-MS and MS/MS), to identify the acylpolyamine toxins contained in the venom of the spiders *Paracoelotes birulai* (Amaurobiidae) [16] and *Agelenopsis aperta* (Agelenidae) [17]. This instrumental setup allowed us to detect and structurally elucidate polyamine derivatives directly, without an additional derivatization step. For instance, a total of 38 compounds were characterized in *A. aperta* [18], and it was shown, too, that for *PA3343* and *PA3334* all acylpentamine isomers of the type *Acyl3334*[3], *Acyl3343*, *Acyl3433*, and *Acyl4333* were present in the toxin mixture. On the basis of this latter finding, we postulated that the biosynthetic precursors of the acylpolyamines in spider venom might be the parent linear polyamines that are 'statistically' mono-acylated at either terminal amino group of the molecule.

To support these assumptions, we searched for free polyamines in the venoms of the forementioned spiders *P. birulai* and *A. aperta*, as well in *Hololena curta* (Agelenidae). Due to the absence of a chromophore on such compounds, UV detection was not appropriate. Reverse-phase HPLC-APCI-MS was selected as the method of choice for the separation and detection of linear polyamines.

Experimental Part

Lyophilized spider venom samples from *A. aperta*, *H. curta*, and *P. birulai* were purchased from *Fauna Laboratories, Ltd.* (Almaty, Kazakhstan). A portion of the crude venom (~100 μg) was dissolved in MeCN/H_2O (3:2) containing 0.1% of

[3] The different acyl groups linked to the polyamine backbones were *4-OH-Bz*, *2,5-(OH)$_2$-Bz*, and *IndAc*.

trifluoroacetic acid (TFA) and the stock solution stored at –20° prior to use. Chromatographic separations were performed with an *Interchim Uptisphere RP C_{18}* column (*UP3HDO–20QS*, 3 μm, 4.6x200 mm; *Intermchim*, Montluçon, France) at a flow rate of 500 μl min^{-1} gradients of MeCN/H_2O/0.1% TFA from 0 to 10% over 5 min and from 10 to 15% over 10 min. The APCI-MS and MS/MS experiments were performed on a *Finnigan TSQ 700* (*Finnigan*, San José, CA, USA) triple quadrupole instrument, and the base peak chromatograms (BPC) were obtained by monitoring the ions with *m*/*z* between 100 and 1000.

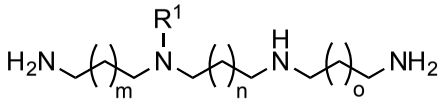
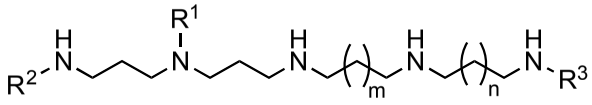
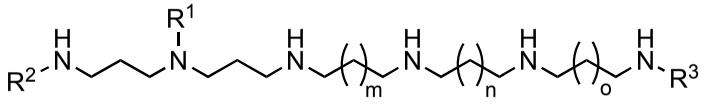
Results and Discussions

The peaks observed in the chromatograms for the venom of the three spider species were distributed over three different regions (*Figure 1*). The first fraction, containing the linear polyamines, eluted with retention times (t_R) close to 5 min. The second fraction, eluting with t_R between 10 and 12 min, was composed of pyrimidine and purine derivatives. This class of compounds was found in the venom of the spider *Latrodectus menavodi* and will not be further discussed here [19]. The third fraction, eluting with t_R between 12 and 15 min, consisted of acetyl- and guanidino-substituted linear polyamines. The polyamine containing first fraction is rather prominent in the venoms of both Agelenidae species *A. aperta* and *H. curta*, but little abundant only in the venom of *P. birulai*. The mass spectra of the compounds co-eluting at t_R = 5.4 min in *A. aperta* (t_R = 5.5 min in the case of *H. curta*) gave rise to the quasi-molecular ions $[M + H]^+$ at *m*/*z* 203, 219, 260, 276, 317, and 333.

Fragmentation by MS/MS of the respective $[M + H]^+$ ions and interpretation of the resulting daughter ions allowed the elucidation of their structure: the series of $[M + H]^+$ ions at *m*/*z* 203, 260, and 317 (the respective masses differing by 57 Da each) correspond to the mixtures of tetramines *PA343* and *PA334*, the pentamines *PA3343* and *PA3334*, and the hexamines *PA33433*, *PA33343*, and *PA33334*, respectively (*Table 1*). The structure assignments are based on the comparison of the MS/MS data of the linear polyamines with those of the parent acylpolyamine compounds and will be discussed separately [20].

The second series of $[M + H]^+$ ions, recorded at *m*/*z* 219, 276, and 333, were identified as N-hydroxylated (+16 Da) derivatives of the tetramines, pentamines, and hexamines mentioned above. However, according to their fragmentation patterns, not all of the theoretically possible isomers of the N-hydroxydes are present in the venoms.

Table 1. Linear Polyamines and Linear Polyamine Derivatives found in the Venom of the Spiders A. aperta, H. curta, *and* P. birulai.

Spider	Trivial Name[a]	t_R [min]	$[M + H]^+$ *m/z*	m	n	o	R[1] [b]	R^2	R^3	Structure
A. aperta *H. curta*	PA343	5.4-5.5	203	1	2	1	H	–	–	
	PA334	5.4-5.5	203	1	1	2	H	–	–	
	PA3(OH)43	5.4-5.5	219	1	2	1	OH	–	–	
	PA3(OH)34	5.4-5.5	219	1	1	2	OH	–	–	
A. aperta *H. curta*	PA3343	5.4-5.5	260	2	1	–	H	H	H	
	PA3334	5.4-5.5	260	1	2	–	H	H	H	
	PA3(OH)343	5.4-5.5	276	2	1	–	OH	H	H	
	PA3(OH)334	5.4-5.5	276	1	2	–	OH	H	H	
	PA3334Gu	12.3	302	1	2	–	H	H	Gu	
P. birulai	PA3(OH)334Gu	12.3	318	1	2	–	OH	H	Gu	
	Ac3334Gu	12.9	344	1	2	–	H	Ac	Gu	
A. aperta *H. curta*	PA33433	5.4-5.5	317	2	1	1	H	H	H	
	PA33343	5.4-5.5	317	1	2	1	H	H	H	
	PA33334	5.4-5.5	317	1	1	2	H	H	H	
	PA3(OH)3343	5.4-5.5	333	1	2	1	OH	H	H	
	PA3(OH)3334	5.4-5.5	333	1	1	2	OH	H	H	
P. birulai	PA33334Gu	12.9	359	1	1	2	H	H	Gu	
	Ac33334Gu	12.9	401	1	1	2	H	Ac	Gu	
H. curta	Ac33343Ac	12.8	401	1	2	1	H	Ac	Ac	

[a]PA stands for polyamine; the figures designate the numbers of methylene units in-between the several N-atoms.
[b]Abbreviations for the substituents: Ac = CH_3CO; Gu = H_2N–C=NH.

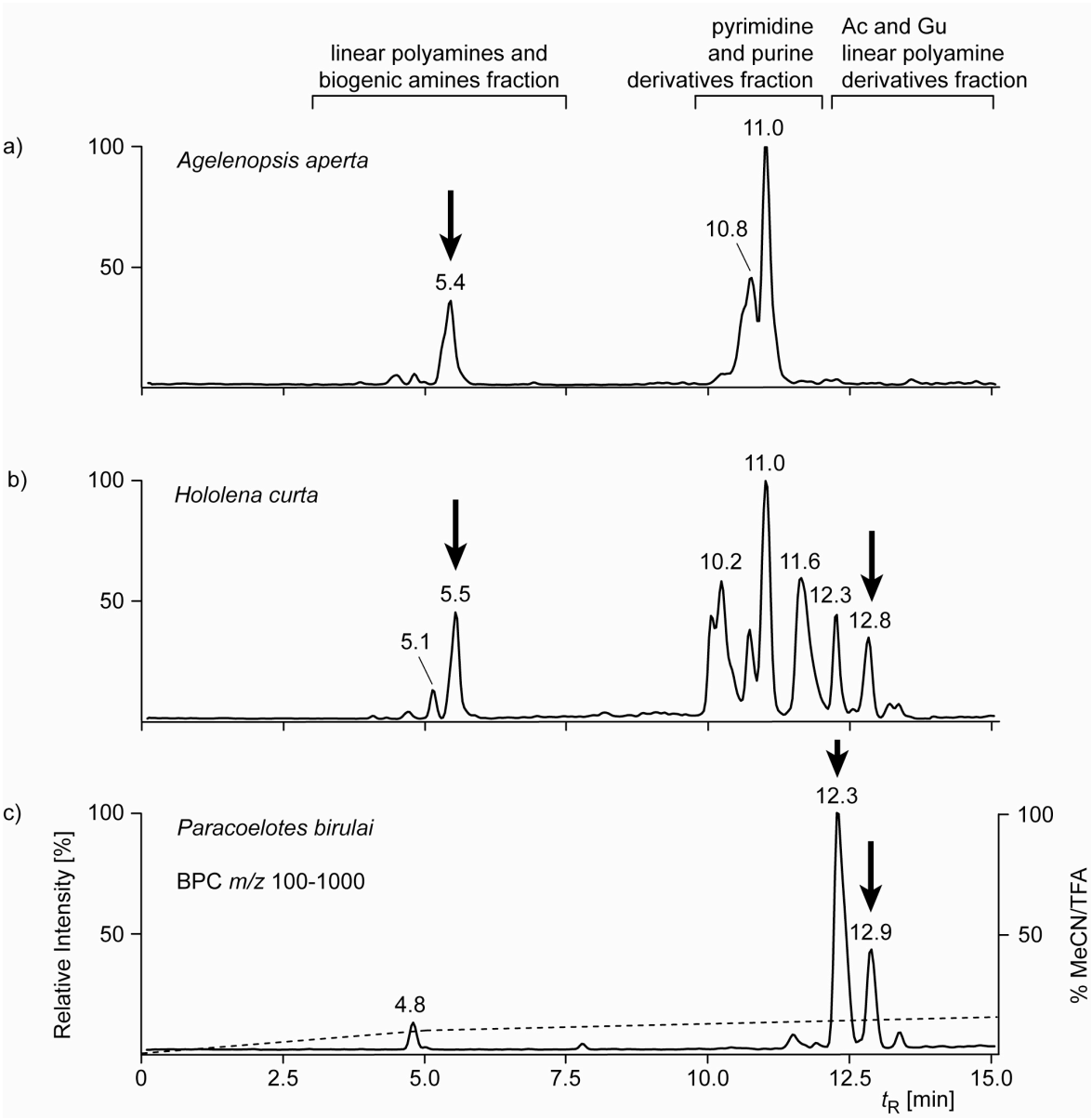


Figure 1. *Reverse-phase HPLC-MS base peak chromatograms (BPC) of the venom of the spiders* A. aperta *(a),* H. curta *(b), and* P. birulai *(c). Fractions containing linear polyamines and derivatives thereof are indicated by an arrow.*

The OH group was solely found to be connected to the secondary N-atoms linked to terminal aminopropyl units. The compounds characterized are *PA3(OH)43* and *PA3(OH)34* for $[M + H]^+$ at *m*/*z* 219, *PA3(OH)343* and *PA3(OH)334* for $[M + H]^+$ at *m*/*z* 276, *PA3(OH)3343* and *PA3(OH)3334* for $[M + H]^+$ at *m*/*z* 333. To our knowledge, no free N-hydroxylated linear polyamines have been isolated from a natural source before.

Concentrating on the acetyl and guanidino derivatives, the chromatogram of the venom of *P. birulai* shows two prominent signals at t_R = 12.3 and 12.9 min. The MS of the respective fractions reveal the presence of $[M + H]^+$ ions at *m*/*z* 302, 318 (t_R = 12.3

min) and at *m/z* 344, 359, and 401 (t_R = 12.9 min) (*Figure 1*c). MS/MS data of the first fraction allowed the deduction of the structures *PA3334Gu*[4] ([M + H]$^+$ *m/z* 302) and the N-hydroxylated derivative *PA3(OH)334Gu* ([M + H]$^+$ *m/z* 318) [20]; the major components of the second fraction were found to be the acetylated and/or guanidylated derivatives *Ac3334Gu* ([M + H]$^+$ *m/z* 344), *PA33334Gu* ([M + H]$^+$ *m/z* 359), and *Ac33334Gu* ([M + H]$^+$ *m/z* 401). The presence of the guanidino group at the terminus of the polyamine backbone was already shown to be characteristic for toxins from *P. birulai* [15]. *H. curta* revealed no guanidino derivatives but a bis-acetylated polyamine, *Ac33343Ac* ([M + H]$^+$ *m/z* 401), eluting with t_R = 12.8 min (*Figure 1*b). Acetylated polyamines were previously isolated solely from bacteria, *e.g.*, N^1-acetylcaldopentamine (*Ac3333*) from *Staphilothermus hellenicus* [21].

Conclusion

The linear polyamines found in the three spider species are supportive for the biosynthesis previously proposed for the acylpolyamines [18]. The linear polyamines arise probably from putrescine (*PA4*) by enzyme catalyzed repetitive addition of aminopropyl units, as described in the literature for mammalian cells [22, 23]. Once the linear polyamines are formed, they can be regiospecifically oxidized to the N-hydroxylated derivatives. Interestingly, some bis-N-hydroxylated acylpolyamine toxins are described for *A. aperta* [17] although no free linear polyamine containing two N–OH moieties could be detected in the present study. Finally, the linear compounds are acylated at either end of the polyamine backbone with one of the known aromatic carboxylic acids *4-OH-BzOH*, *2,5-(OH)$_2$-BzOH*, *2,4-(OH)$_2$-PhAcOH*, *IndAcOH*, *4-OH-IndAcOH*, and *6-OH-IndAcOH* [1] to yield the acylpolyamines.

As already mentioned, underivatized linear free polyamines are of low abundance in the case of *P. birulai*. All of the four compounds detected for this species possess a guanidino end group attached to the aminobutyl portion of the polyamine backbone. However, 'regular' acylpolyamine toxins, having no guanidino end group, are present in the venom cocktail of *P. birulai* [16]. Elucidation of the complete biosynthetic route to acylpolyamines has yet to be confirmed, *e.g.*, by *in-vivo* experiments.

[4] Gu designates a terminal guanidino group instead of a primary amine.

References

[1] A. Schäfer, H. Benz, W. Fiedler, A. Guggisberg, S. Bienz, M. Hesse, *Alkaloids* **1994**, *45*, 1.

[2] S. Chesnov, L. Bigler, M. Hesse, *Eur. J. Mass Spectrom.* **2002**, *8*, 1.

[3] Y. Itagaki, T. Nakajima, *J. Toxicol. Toxin Reviews* **2000**, *19*, 23.

[4] S. Bienz, R. Detterbeck, C. Ensch, A. Guggisberg, U. Häusermann, C. Meisterhans, B. Wendt, C. Werner, M. Hesse, *Alkaloids* **2002**, *58*, 83.

[5] C. Lange, C. Paris, M.-L. Celerier, *Rapid Commun. Mass Spectrom.* **1992**, *6*, 289.

[6] C. Lange, C. Paris, M.-L. Celerier, *Rapid Commun. Mass Spectrom.* **1992**, *6*, 517.

[7] P. H. Duffield, A. M. Duffield, P. R. Carroll, D. Morgans, *Biomed. Mass Spectrom.* **1979**, *6*, 105.

[8] S. G. Cabbiness, C. W. Gehrke, K. C. Kuo, T. K. Chan, J. E. Hall, S. A. Hudiburg, G. V. Odell, *Toxicon* **1980**, *18*, 681.

[9] K. Hagiwara, A. Tokita, A. Miwa, N. Kawai, Y. Murata, A. Uchida, T. Nakajima, *Jpn. J. Sanit. Zool.* **1991**, *42*, 77.

[10] K. Hamana, M. Niitsu, K. Samejima, S. Matsuzaki, *Comp. Biochem. Physiol.* **1991**, *100B*, 399.

[11] T. Oshima, S. Kawahata, *J. Biochem.* **1983**, *93*, 1455.

[12] K. Hamana, M. Niitsu, K. Samejima, S. Matsuzaki, *FEMS Microbiol. Lett.* **1990**, *68*, 27.

[13] K. Hamana, M. Niitsu, S. Matsuzaki, K. Samejima, Y. Igarashi, T. Kodama, *Biochem. J.* **1992**, *284*, 741.

[14] M. Niitsu, K. Samejima, S. Matsuzaki, K. Hamana, *J. Chromatogr.* **1993**, *641*, 115.

[15] K. Hamana, M. Niitsu, K. Samejima, T. Itoh, H. Hamana, T. Shinozawa, *Microbios* **1998**, *93*, 7.

[16] S. Chesnov, L. Bigler, M. Hesse, *Helv. Chim. Acta* **2000**, *83*, 3295.

[17] S. Chesnov, L. Bigler, M. Hesse, *Helv. Chim. Acta* **2001**, *84*, 2178.

[18] N. Manov, M. Tzouros, S. Chesnov, L. Bigler, S. Bienz, *Helv. Chim. Acta* **2002**, *85*, 2827.

[19] A. Horni, D. Weickmann, M. Hesse, *Toxicon* **2000**, *39*, 425.

[20] M. Tzouros, S. Chesnov, S. Bienz, M. Hesse, L. Bigler, in prep.

[21] K. Hamana, T. Tanaka, R. Hosoya, M. Niitsu, T. Itoh, *J. Gen. Appl. Microbiol.* **2003**, *49*, 287.

[22] H. Geneste, M. Hesse, *Chemie in unserer Zeit* **1998**, *32*, 206.

[23] C. W. Tabor, H. Tabor, *Ann. Rev. Biochem.* **1984**, *53*, 749.

CHAPTER 4

Two New Spermidine Alkaloids from *Chisocheton weinlandii*[1]

Abstract

The investigation of the MeOH extract of the leaves of *Chisocheton weinlandii* HARMS (Meliaceae) revealed two new open-chain spermidine alkaloids, chisitine 1 (**1**) and chisitine 2 (**2**). Their structures were elucidated by NMR spectroscopy, tandem-mass spectrometry, and independent syntheses. Detailed MS/MS fragmentation pathways are discussed for both compounds based on H/D exchange and ^{18}O-labeling experiments.

Introduction

Polyamine alkaloids are found in a large variety of natural sources including plants and insects. These natural products are classified into two major categories depending on whether the structure is cyclic or open-chained [1]. In general, such polyamine alkaloids are composed of a putrescine (= butane-1,4-diamine), spermidine (= *N*-(3-aminopropyl)butane-1,4-diamine), or spermine (= *N*,*N'*-bis(3-aminopropyl)butane-1,4-diamine) backbone that carries one or more substituents at the N-atoms, mostly acyl groups. Their determination was usually performed by a combination of several analytical techniques, the methods of choice being mass spectrometry combined with NMR spectroscopy.

However, NMR spectroscopy often does not securely assess the exact framework of the polyamine portion within these alkaloids because of signal overlap of the methylene protons. Furthermore, acyl-substituted polyamines display an additional range of complexity in their NMR spectra due to s-*cis*/s-*trans* isomerism of the amide functions. The NMR spectra of such isomer mixtures, exhibiting multiple signals for the NMR active nuclei, always leave doubt about whether the sample was pure or not.

[1] M. Tzouros, L. Bigler, S. Bienz, M. Hesse, A. Inada, H. Murata, Y. Inatomi, T. Nakanishi, D. Darnaedi, *Helv. Chim. Acta* **2004**, *87*, 1411.

Mass spectrometry — in combination with tandem-mass spectrometry (MS/MS) — is gaining more and more importance in the identification and characterization of polyamine alkaloids. Due to their high basicity and low volatility, polyamines are excellent candidates for mass-spectrometric identification by means of electrospray ionization (ESI-MS). This method allows to delimite the number of N-atoms present in the molecule. In addition, MS/MS experiments can provide precious information on the polyamine backbones and on the attachment sites of substituents. Furthermore, as compared to NMR spectroscopy, data acquisition for MS is much faster, and requires considerably smaller amounts of material.

In connection with our ongoing search for new polyamine alkaloids from plants and animal sources, we were interested in the MeOH extracts of the leaves of *Chisocheton weinlandii* HARMS (Meliaceae). This tree is distributed throughout southern Irian Jaya (Papua New Guinea, Indonesia). Around fifty putrescine alkaloids have already been characterized from species belonging to the plant family of Meliaceae [1]. Several *Chisocheton* species have been screened for their natural-product content; among them, *Chisocheton paniculatus* HIERN has been intensively investigated. Five apo-tirucallol derivatives and four tetranortriterpenoids from the wood and seeds of *C. paniculatus* were identified [2]. Four meliacin-type compounds were found in the fruits [3], and five protolimonoids (called paniculatin B, C, D, G, and H) and one limonoid (named arunachalin) were isolated and identified from the mature wood of the plant [4]. For *C. weinlandii*, on the other hand, no study of the chemical constituents is found in the literature. Only some insecticidal activity was reported for extracts of its leaves and twigs [5]. These extracts showed inhibition to larval growth of the variegated cutworm *Peridroma saucia* HÜBNER (Noctuinidae). We have now studied the constituents of the leaves of the plant and isolated the two new spermidine-type alkaloids **1** and **2** (see *Figure 1*). Their structures were established on the basis of NMR spectroscopy, tandem-mass spectrometry, and comparison with their synthetic equivalents.

Results and Discussion

Isolation

Extraction of the crushed leaves of *C. weinlandii* with MeOH and distribution of the extract between H_2O and AcOEt yielded a crude mixture that was separated by column chromatography (silica gel). With the eluent $CHCl_3$/MeOH 5:1, a mixture was obtained that was further subjected to semi-preparative reversed-phase HPLC, resulting in the isolation of two pure compounds named chisitine 1 (**1**) and chisitine 2 (**2**) (*Figure 1*). To the best of our knowledge, these are the first spermidine alkaloids isolated from a Meliaceae species.

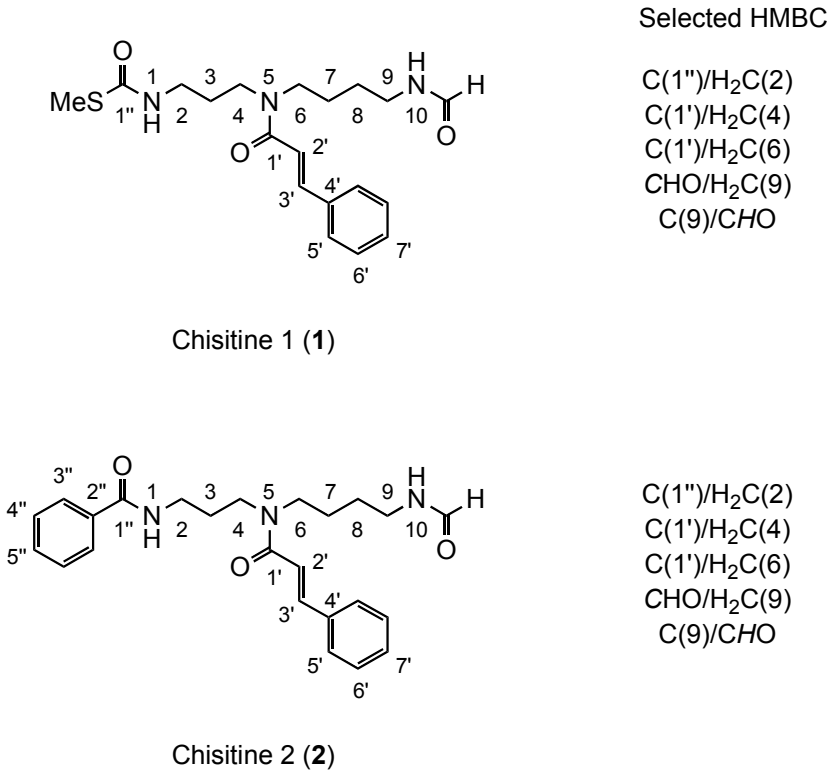


Figure 1. *Structure and selected HMBC data of chisitine 1 (**1**) and 2 (**2**).*[2]

Structure Elucidation

Chisitine 1 (**1**) shows in its ESI-MS a series of three peaks at *m/z* 378 ($[M + H]^+$), 400 ($[M + Na]^+$), and 416 ($[M + K]^+$) that allow the assignment of its relative molecular mass as 377. The value suggests the presence of an odd number of N-atoms in the molecule. The ^{1}H- and ^{13}C-NMR spectra of **1**, showing a 1:1 ratio of two isomers (for

[2] Arbitrary numbering; for systematic name, see Experimental Part.

data, see isomers **1a**, and **1b** in *Tables 1* and *2*), indicate the presence of a cinnamoyl (= (2*E*)-3-phenylprop-2-enoyl) substituent. In the ^{1}H-NMR spectrum of **1**, the two doublets at *δ* 7.10, 7.05 and the broad doublet at *δ* 7.49 (^{3}J = 15.4) are attributed to the olefinic protons H–C(2′) and H–C(3′) of the two isomeric forms **1a** and **1b**. The corresponding ^{13}C-NMR signals for C(2′) and C(3′) are found at *δ* 118.4 and 118.3, and 141.3 and 141.2 and secured by HSQC. The signals at *δ*(C) 161.0, 160.9 (for **1a** and **1b**) and the broad singulet at *δ*(H) 8.00 suggest the presence of a formamide function [6]. A third substituent is identified by the signal at *δ*(H) *ca*. 2.20 (3 H), which correlates to the quadruplet (determined by DEPT) at *δ*(C) 11.5 (two overlapping signals). These signals are attributed to the unusual MeSC(O)N residue. The analytical data are in accordance with other carbamothioate-containing alkaloids [7, 8].

The presence of the MeSC(O)N group is further supported by the MS/MS results: the intense fragment ion **l** (*m*/*z* 330), corresponding to the loss of 48 Da from the quasi-molecular ion ($[M + H]^{+}$) at *m*/*z* 378, indicates an easy loss of MeSH (see *Figure 3*a, in *Tandem-Mass Spectrometry of Chisitines 1 and 2*). ^{1}H-NMR Signals registered between *δ* 1.36 and 3.49 ppm (integrating for 14 H) and the ^{13}C-NMR signals found between *δ* 26.3 and 46.8 ppm (all triplets) suggest a spermidine backbone for the compound. The sites of attachment of the three substituents at the polyamine core, as well as the detailed assignments of the signals, were finally established on the basis of the MS/MS analysis (see next paragraph) as well as on the basis of the HMBC experiment (selected HMBC data in *Figure 1*). After detailed analysis of further NMR and MS data, the structure of chisitine 1 (**1**) was elucidated as *S*-methyl [3-{[4-(formylamino)butyl][(2*E*)-3-phenylprop-2-enoyl]amino}propyl]carbamothioate. This compound was synthesized, and the synthetic product was found to be identical with the natural product with respect to all its spectroscopic data (see *Synthesis of chisitine 1 and 2*).

The second compound, chisitine 2 (**2**), shows three adduct ions in the ESI-MS at *m*/*z* 408 ($[M + H]^{+}$), 430 ($[M + Na]^{+}$), and 446 ($[M + K]^{+}$), consistent with a relative molecular mass of 407. This indicates that chisitine 2 also contains an odd number of N-atoms. The ^{1}H- and ^{13}C-NMR spectra of this alkaloid show the presence of the same 1:1 ratio of two isomers as found for **1** (for data, see isomers **2a** and **2b** in *Tables 1* and *2*).

Table 1. *[1]H-NMR Data (500.1 or 600.1 MHz, (D_6)DMSO) of Chisitines 1 (**1**) and 2 (**2**). δ in ppm, J in Hz.*

	1[a])		**2**[b])	
	Isomer **1a**	Isomer **1b**	Isomer **2a**	Isomer **2b**
H–N(1)	8.23 (br. *s*)	8.11 (br. *s*)	8.49–8.47 (*m*)	8.57–8.55 (*m*)
CHO	8.00 (br. *s*)[c])		8.00 (br. *s*)[c])	
H–N(10)				
H–C(3'')	—		7.87 (*d*, *J* = 7.4)	7.85 (*d*, *J* = 7.4)
H–C(5')	7.70–7.68 (*m*)[c])		7.71 (*d*, *J* = 7.4)	7.58 (*d*, *J* = 7.1)
H–C(5'')	—			
H–C(3')	7.49 (br. *d*, *J* ≈ 15.2)[c])		7.54–7.45 (*m*)[c])	
H–C(4'')	—			
H–C(6')	7.44–7.36 (*m*)[c])		7.44–7.27 (*m*)[c])	
H–C(7')				
H–C(2')	7.10 (*d*, *J*= 15.4)	7.05 (*d*, *J*= 15.4)	7.13 (*d*, *J* = 15.4)	7.05 (*d*, *J* = 15.4)
CH_2(4)	3.49–3.45 (*m*)[c])	3.32–3.29 (*m*)[c])	3.55–3.50 (*m*)[c])	3.44–3.42 (*m*)[c])
CH_2(6)	3.32–3.29 (*m*)[c])	3.49–3.45 (*m*)[c])	3.38–3.34 (*m*)[c])	3.55–3.50 (*m*)[c])
CH_2(2)	3.21–3.15 (*m*)[c])	3.13–3.08 (*m*)[c])	3.38–3.34 (*m*)[c])	3.29–3.26 (*m*)[c])
CH_2(9)	3.13–3.08 (*m*)[c])		3.13–3.08 (*m*)[c])	
MeS	2.21, 2.20 (2 *s*)[d])		—	
CH_2(3)	1.74–1.71 (*m*)	1.68–1.65 (*m*)	1.87–1.84 (*m*)	1.79–1.77 (*m*)
CH_2(7)	1.54–1.36 (*m*)[c])		1.57–1.49 (*m*)[c])	
CH_2(8)			1.38–1.41 (*m*)	1.48–1.43 (*m*)

[a]) 500.1 MHz. [b]) 600.1 MHz. [c]) Signals of isomers **a** / **b** overlapped. [d]) Not distinguishable.

Moreover, the data also indicate the presence of an acyl-substituted spermidine, carrying the cinnamoyl and formyl moieties, but not the methylcarbamothioate unit. Since the sites of attachment of the groups are identical to those found for chisitine 1, the only difference between **2** and **1** concerns the nature of one substituent. The additional signals in the aromatic region of the ^{13}C-NMR spectra of **2** at *δ* 134.7, 134.5 (two singulets, C(2'')), 131.1 and 131.0 (two doublets, C(5'')), 128.3 (two overlapping doublets, C(4'')), and 127.1 and 127.0 (two doublets, C(3'')) are assigned to a benzoyl group [9]. The replacement of the MeSCO in **1** by the PhCO group in **2** is consistent with the difference of 30 Da in their relative molecular mass. The sites of attachment of the substituents at the spermidine core for both isomers were established on the basis of a detailed MS/MS analysis (see the following paragraph) and of the HMBC spectra (see data in *Figure 1*). Based on further spectral data, the structure of **2** was

determined to be *N*-{3-{[4-(formylamino)butyl][(2*E*)-3-phenylprop-2-enoyl]amino}-propyl}benzamide and confirmed by comparison with the analytical data of the synthetic chisitine 2 (see *Synthesis of Chisitine 1 and 2*).

Table 2. *^{13}C-NMR Data (125.8 or 150.9 MHz, (D_6)DMSO) of Chisitines 1 (**1**) and 2 (**2**). δ in ppm, J in Hz.*

	1[a])		**2**[b])	
	Isomer **1a**	Isomer **1b**	Isomer **2a**	Isomer **2b**
C(1′′)	166.2 (*s*)	166.0 (*s*)	166.4 (*s*)	166.1 (*s*)
C(1′)	165.2 (*s*)	164.9 (*s*)	165.3 (*s*)	164.9 (*s*)
CHO	161.0 (*d*)	160.9 (*d*)	161.0 (*d*)	160.9 (*d*)
C(3′)	141.3 (*d*)	141.2 (*d*)	141.3 (*d*)	141.2 (*d*)
C(4′)	135.1 (*s*)	135.0 (*s*)	135.1(*s*)	135.0 (*s*)
C(2′′)	—	—	134.7 (*s*)	134.5 (*s*)
C(5′′)	—	—	131.1 (*d*)	131.0 (*d*)
C(7′)	129.5 (*d*)[c])		129.5 (*d*)	129.4 (*d*)
C(6′)	128.7 (*d*)[c])		128.7 (*d*)	128.6 (*d*)
C(4′′)	—	—	128.3 (*d*)[c])	
C(5′)	127.9 (*d*)[c])		128.0 (*d*)	127.8 (*d*)
C(3′′)	—	—	127.1 (*d*)	127.0 (*d*)
C(2′)	118.4 (*d*)	118.3 (*d*)	118.5 (*d*)	118.4 (*d*)
C(6)	45.4 (*t*)	46.8 (*t*)	45.4 (*t*)	46.8 (*t*)
C(4)	44.7 (*t*)	43.6 (*t*)	45.0 (*t*)	43.6 (*t*)
C(2)	38.1 (*t*)	38.5 (*t*)	36.7 (*t*)	36.9 (*t*)
C(9)	36.8 (*t*)	36.6 (*t*)	36.8 (*t*)	36.6 (*t*)
C(3)	29.5 (*t*)	27.7 (*t*)	29.6 (*t*)	27.7 (*t*)
C(8)	24.9 (*t*)	26.7 (*t*)	25.0 (*t*)	26.7 (*t*)
C(7)	26.6 (*t*)	26.3 (*t*)	26.6 (*t*)	26.3 (*t*)
MeS	11.5 (*q*)[c])		—	—

[a]) 125.8 MHz. [b]) 150.9 MHz. [c]) Signals of isomers **a**/**b** overlapped.

Tandem-Mass Spectrometry of Chisitines 1 and 2

A special problem arises in the correct placing of the two substituents (formyl and (methylthio)carbonyl in case of **1** and formyl and benzoyl in case of **2**) at the two primary N-atoms of the spermidine core. The question that arises is whether the amino groups are separated by three or four CH_2 residues from the third central N-atom. In addition to our earlier investigations in this field of isomerism [10-12], we

have studied very carefully this problem. Indeed, it seems necessary to determine the general characteristic MS fragmentation of this kind of compounds, because similar natural products isolated in much smaller amounts have to be investigated in the future.

Compounds **1** and **2** were examined by ESI-MS/MS on a quadrupole ion-trap mass spectrometer. The two compounds produce two generations of daughter ions (MS^2 and MS^3) on the basis of which fragmentation mechanisms are proposed. The aim of their detailed elucidation is to detect characteristic fragmentation pathways that are diagnostic for the structures proposed for **1** and **2**. Characteristic ions could indicate the location of the substituents as well as the sequence of the polyamine backbone (*PA34* or *PA43*). The MS/MS data of compound **2** contain more informative ion responses allowing the postulation of general fragmentation pathways, and are, thus, discussed first.

Upon MS^2, quasi-molecular ion $[\mathbf{2} + H]^+$ at *m*/*z* 408 yields the four important fragment ions **a**, **d**, and **e**/**e'** at *m*/*z* 390, 278, and 260 (*Figure* 2a). These ions are believed to arise from two major *Pathways A* and *B* (*Scheme 1*). Following *Pathway A*, $[\mathbf{2} + H]^+$ could lose the cinnamoyl moiety (–130 Da) to form fragment ion **d** (*m*/*z* 278). This suggested four-center cleavage has already been reported for *N*-coumaroylputrescine [12]. Fragment ion **d** could lose H_2O by reaction of the secondary amine (N(5)) with either one of the two amide groups to form cyclic fragment ions **e** (1,6-cyclization) or **e'** (1,7-cyclization), detected at *m*/*z* 260 as the base peaks in MS^2. Analogous gas-phase dehydrations involving the amide O-atom have been observed previously with peptides [13]. The cyclization **d** → **e** and **e'** is established by investigation of the labeled analog [^{18}O]-**2** (*Figure* 2c). The MS^2 of [[^{18}O]-**2** + H]$^+$ (*m*/*z* 410) reveals a doublet of peaks corresponding to **e** at *m*/*z* 260 and [^{18}O]-**e'** (*m*/*z* 262), which attests that both proposed pathways are followed concurrently.

Isolation and further fragmentation (MS^3) of **e** and **e'** at *m*/*z* 260 yield the four new informative ions **g**, **h**, **j**, and **k** at *m*/*z* 162, 161, 105, and 99 respectively. Probably ion **e'** leads to two cyclic fragment ions **g** (*m*/*z* 162) and **k** (*m*/*z* 99), and to the benzoyl ion **j** (*m*/*z* 105). These three ions are established to originate from **e'** by investigation of [^{18}O]-**2** (see *Figure* 2c): the corresponding signals for **g** and **j** are at *m*/*z* 164 (+2 Da) and 107 (+2 Da), respectively, whereas ion **k** is at the same *m*/*z* value 99 (see *Scheme 1*). Fragment ion **g** (*m*/*z* 162) reveals that the benzoyl group is linked to the trimethylene portion of the spermidine, while **k** (*m*/*z* 99) attests that the formyl moiety is located at

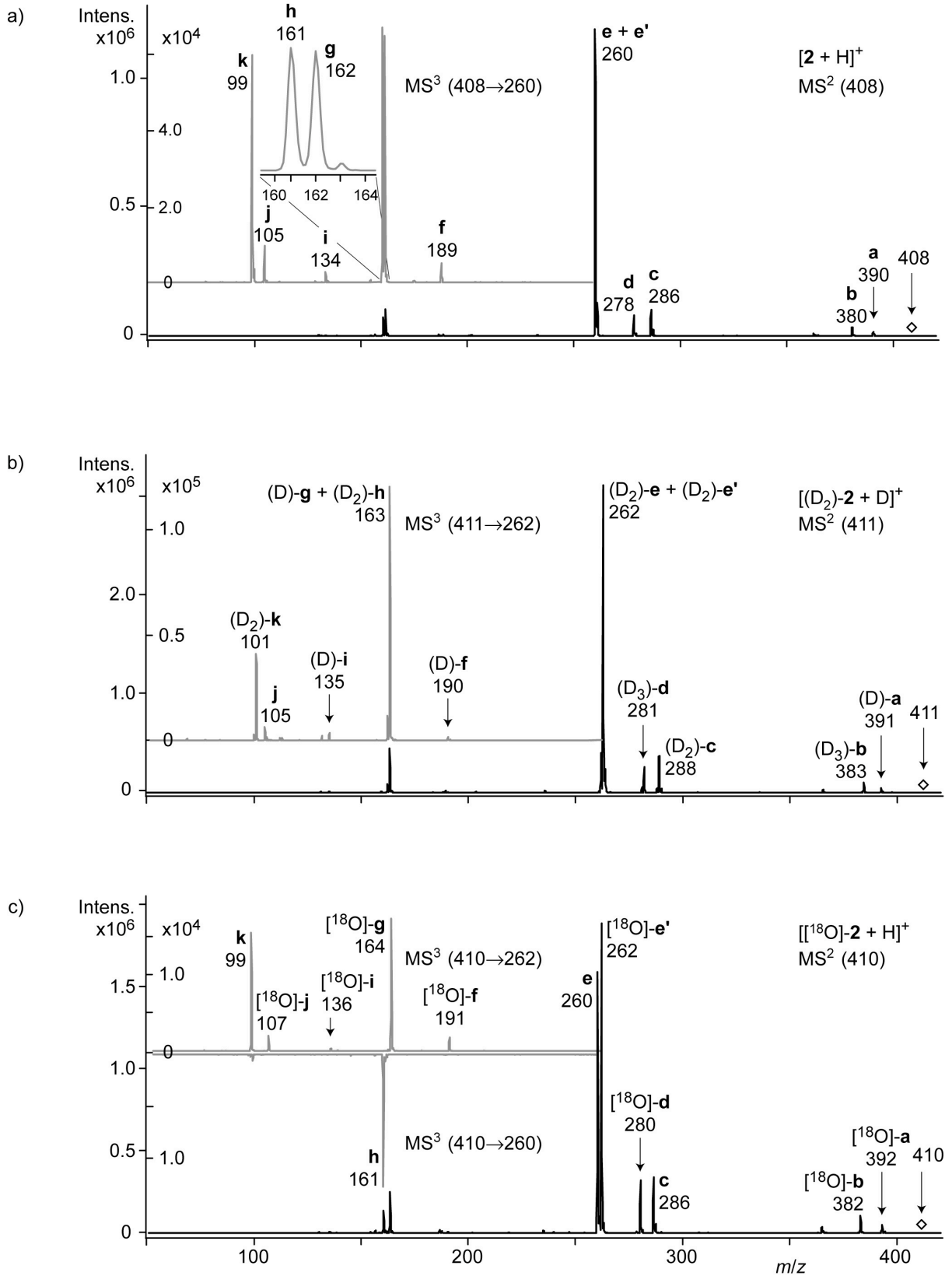
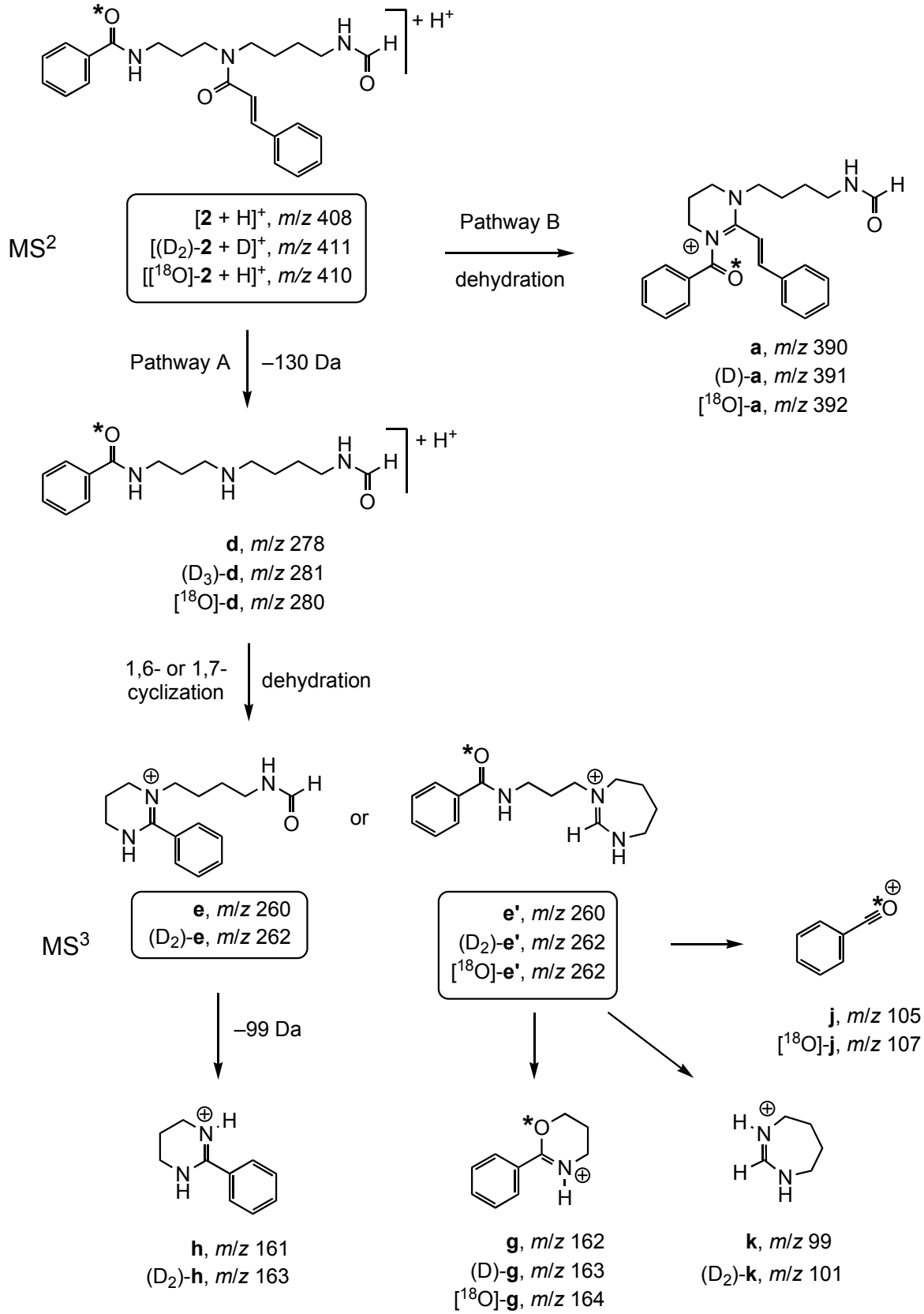


Figure 2. *MS^n plots of the quasi-molecular ions of chisitine 2 (**2**): a) MS^2 (408) (black line) and MS^3 (408→260) (grey line) of $[2 + H]^+$, b) MS^2 (411) (black line) and MS^3 (411→262) (grey line) of $[(D_2)\text{-}2 + D]^+$, and c) MS^2 (410) (black line) and MS^3 (410→262)/(410→260) (grey lines) of $[[^{18}O]\text{-}2 + H]^+$. Fragment ions are designated according to decreasing* m/z *values (**a** to **k**).*



Scheme 1. *MS fragmentation of the quasi-molecular ions of chisitine 2 (**2**).*[3]

[3] The * denotes the location of the ^{18}O-atom in the case of the labeled analog [^{18}O]-**2**. The boxes indicate the selected *m*/*z* values submitted to MS^2 and MS^3. The structures of the fragment ions **b**, **c**, **f**, and **i** are not depicted.

the end of the tetramethylene unit. Fragmentation of the six-membered ion **e** (*m*/*z* 260) leads to the formation of ion **h** recorded at *m*/*z* 161, additionally supported by the MS^3 (410 → 260) of 'pure' **e** in the spectrum of [^{18}O]-**2** (*Figure 2*c). The diagnostic ions **g**, **k**, and **h**, therefore, justify the structure of chisitine 2 (**2**) independently of the NMR spectra.

The second important fragmentation *Pathway B* starts with the direct dehydration of quasi-molecular ion [**2** + H]$^+$ giving rise to a signal at *m*/*z* 390 ([**2** + H – 18]$^+$). Again, the loss of H_2O is explained by the formation of a cyclic structure, fragment ion **a** (see *Scheme 1*). The fragment ions **b** (*m*/*z* 380), **c** (*m*/*z* 286), **f** (*m*/*z* 189), and **i** (*m*/*z* 134) (*Figure 2*a) are not discussed because they are not characteristic for the structure elucidation of **2**.

To support the proposed mechanism, an H/D-exchange experiment was performed. Thus, **2** was dissolved in an acidic solution of MeOD (0.1% of DCl), and the resulting deuterated compound [(D_2)-**2** + D]$^+$ (*m*/*z* 411) was investigated by ESI-MS and MS/MS (*Figure 2*b). The mass shifts observed of the fragment ions, which depend on the number of their exchangeable protons, support the proposed mechanism in *Scheme 1*. All fragment ions are summarized in *Table 3*.

In the case of chisitine 1 (**1**), the presence of the (methylthio)carbonyl substituent instead of the benzoyl group of **2** has dramatic consequences on the MS behavior of **1** (*Figure 3*). The S-containing group dominates the fragmentation paths (*Scheme 2*), which results in less information to be extracted from the MS/MS data as compared to those obtained with chisitine 2 (**2**). *Pathway A*, which begins with the cleavage of the cinnamoyl group (–130 Da) and is followed by a dehydration reaction (–18 Da) to form the two isomeric cyclic fragment ions **n** and **n'** at *m*/*z* 230 (in analogy to the ions **e** and **e'** in the case of **2**), is clearly unfavored (*Figure 3* and *Scheme 2*). Despite the low intensity of the ions obtained, *Pathway A* is taking place and is attested by the formation of fragment ions **p** (*m*/*z* 182) when **n** and **n'** are submitted to an MS^3 experiment (spectra not shown).

Quasi-molecular ion [**1** + H]$^+$ (*m*/*z* 378) does not undergo dehydration (*Pathway B*) but loses MeSH (–48 Da), following the favored *Pathway C*, to form the intense fragment ion **l** at *m*/*z* 330 (base peak in MS^2) (*Scheme 2*). The observation can be rationalized by the fact that MeSH is a better leaving group than H_2O.

Table 3. *Fragment Ions in the MS^n Spectra of Chisitine 1 (**1**) and 2 (**2**).*

	Quasi-molecular ion		MS^n Experiment	Fragment ions, m/z (relative intensity)
	Type	m/z		
1	$[\mathbf{1} + H]^+$	378	MS^2 (378)	**l**, 330(100); **m**, 302(1); **n**/**n′**, 230(1); **o**, 200(1); **p**, 182(14); **q**, 155(3)
			MS^3 (378→330)	**m**, 302(4); **o**, 200(6); **p**, 182(100); **q**,155(4); **r**, 131(4)
(D_2)-**1**	$[(D_2)\text{-}\mathbf{1} + D]^+$	381	MS^2 (381)	(D_2)-**l**, 332(100); (D_2)-**m**, 304(1); (D_2)-**n**/(D_2)-**n′**, 232(2); (D_2)-**o**, 202(4); (D)-**p**, 183(14); (D)-**q**, 156(2)
			MS^3 (381→332)	(D_2)-**m**, 304(6); (D_2)-**o**, 202(7); (D)-**p**, 183(100); (D)-**q**, 156(1); **r**, 131(4)
2	$[\mathbf{2} + H]^+$	408	MS^2 (408)	**a**, 390(2); **b**, 380(2); **c**, 286(9); **d**, 278(6); **e**/**e′**, 260(100); **g**, 162(9); **h**, 161(6)
			MS^3 (408→260)	**f**, 189(9); **g**, 162(97); **h**, 161(100); **i**, 134(4); **j**, 105(14); **k**, 99(84)
(D_2)-**2**	$[(D_2)\text{-}\mathbf{2} + D]^+$	411	MS^2 (411)	(D)-**a**, 391(2); (D_3)-**b**, 383 (3); (D_2)-**c**, 288(12); (D_3)-**d**, 281(8); (D_2)-**e**/(D_2)-**e′**, 262(100); (D)-**g**/(D_2)-**h**, 163(14)
			MS^3 (411→262)	(D)-**f**, 190(2); (D)-**g**/(D_2)-**h**, 163(100); (D)-**i**, 135(3); **j**, 105(5); (D_2)-**k**, 101(34)
$[^{18}O]$-**2**	$[[^{18}O]\text{-}\mathbf{2} + H]^+$	410	MS^2 (410)	$[^{18}O]$-**a**, 392(3); $[^{18}O]$-**b**, 382(6); **c**, 286(18); $[^{18}O]$-**d**, 280(17); $[^{18}O]$-**e′**, 262(100); **e**, 260(84); $[^{18}O]$-**g**, 164(13); **h**, 161(7)
			MS^3 (410→262)	$[^{18}O]$-**f**, 191(10); $[^{18}O]$-**g**, 164(100); $[^{18}O]$-**i**, 136(1); $[^{18}O]$-**j**, 107(11); **k**, 99(89)
			MS^3 (410→260)	**h**, 161(100)

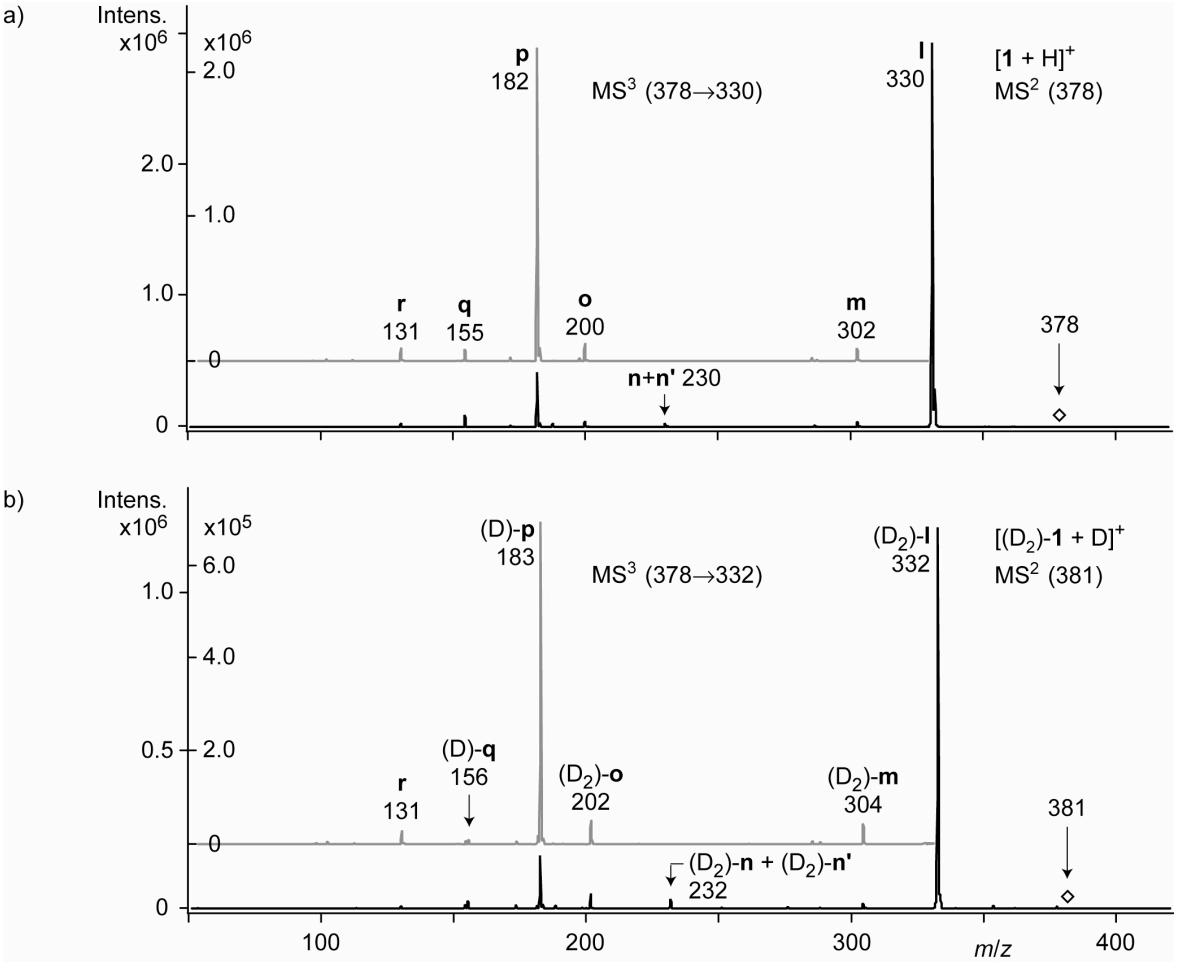
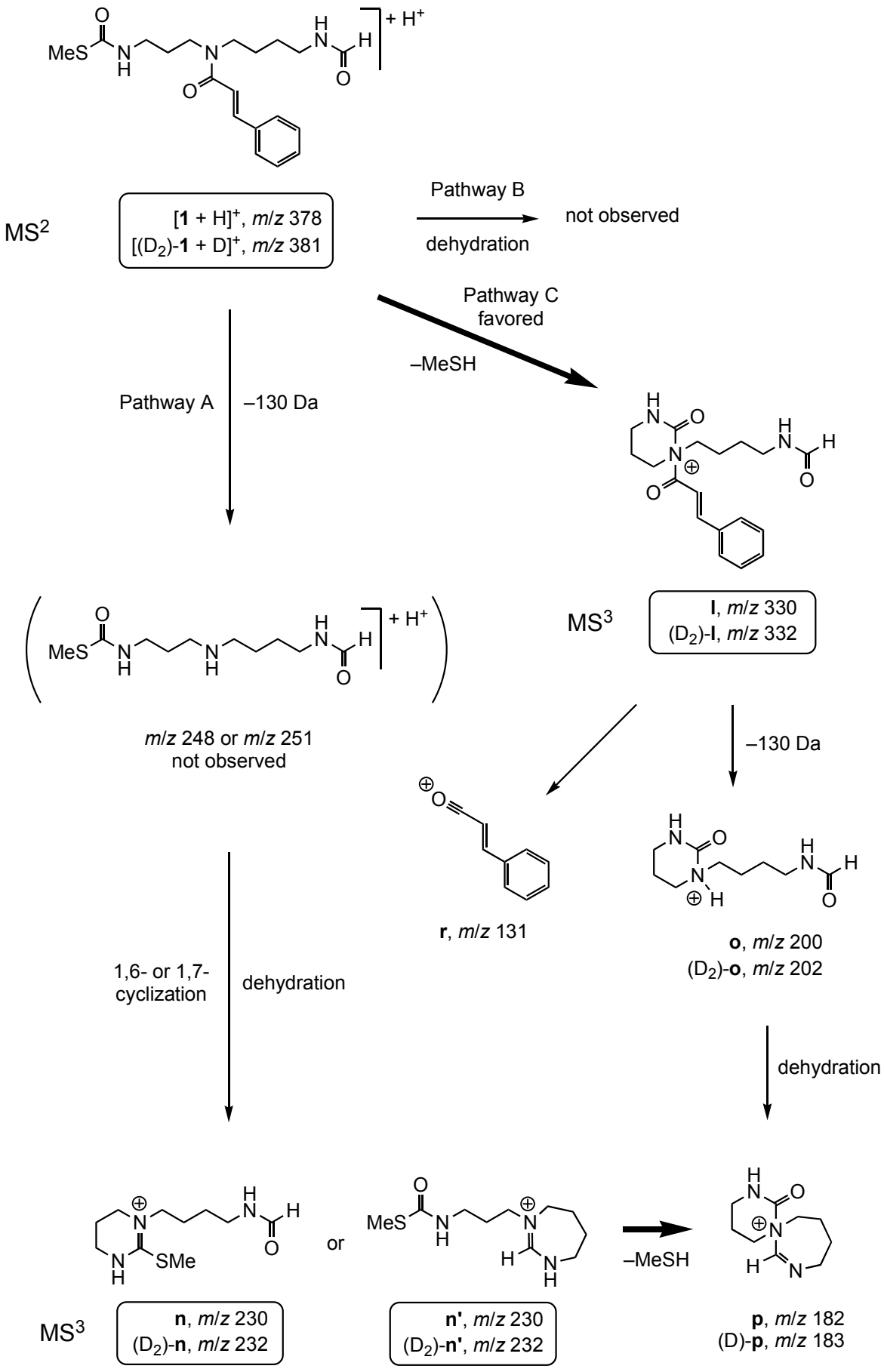


Figure 3. *MS^n plots of the quasi-molecular ions of chisitine 1 (**1**): a) MS^2 (378) (black line) and MS^3 (378→330) (grey line) of [**1** + H]$^+$, and b) MS^2 (381) (black line) and MS^3 (381→332) (grey line) of [(D_2)-**1** + D]$^+$. Fragment ions are designate according to decreasing* m/z *value (**l** to **r**).*

Isolation and further fragmentation of ion **l** (*m*/*z* 330) leads to the loss of the cinnamoyl moiety (–130 Da) under formation of fragment ion **o** (*m*/*z* 200). Subsequent dehydration gives bicyclic ion **p**, recorded at *m*/*z* 182 (base peak in MS^3). The latter ion can also derive from ion **n'** (*m*/*z* 230) by expulsion of MeSH (–48 Da). In addition, fragment ion **l** (*m*/*z* 330) produces the cinnamoyl cation **r** (*m*/*z* 131). Analogously to the above mentioned H/D-exchange experiment, compound **1** was treated in the same way to give deuterated analog [(D_2)-**1** + D]$^+$ (*m*/*z* 381). The ion was investigated by ESI-MS and MS/MS (*Figure 3*b) to support the mechanism proposed in *Scheme 2*.

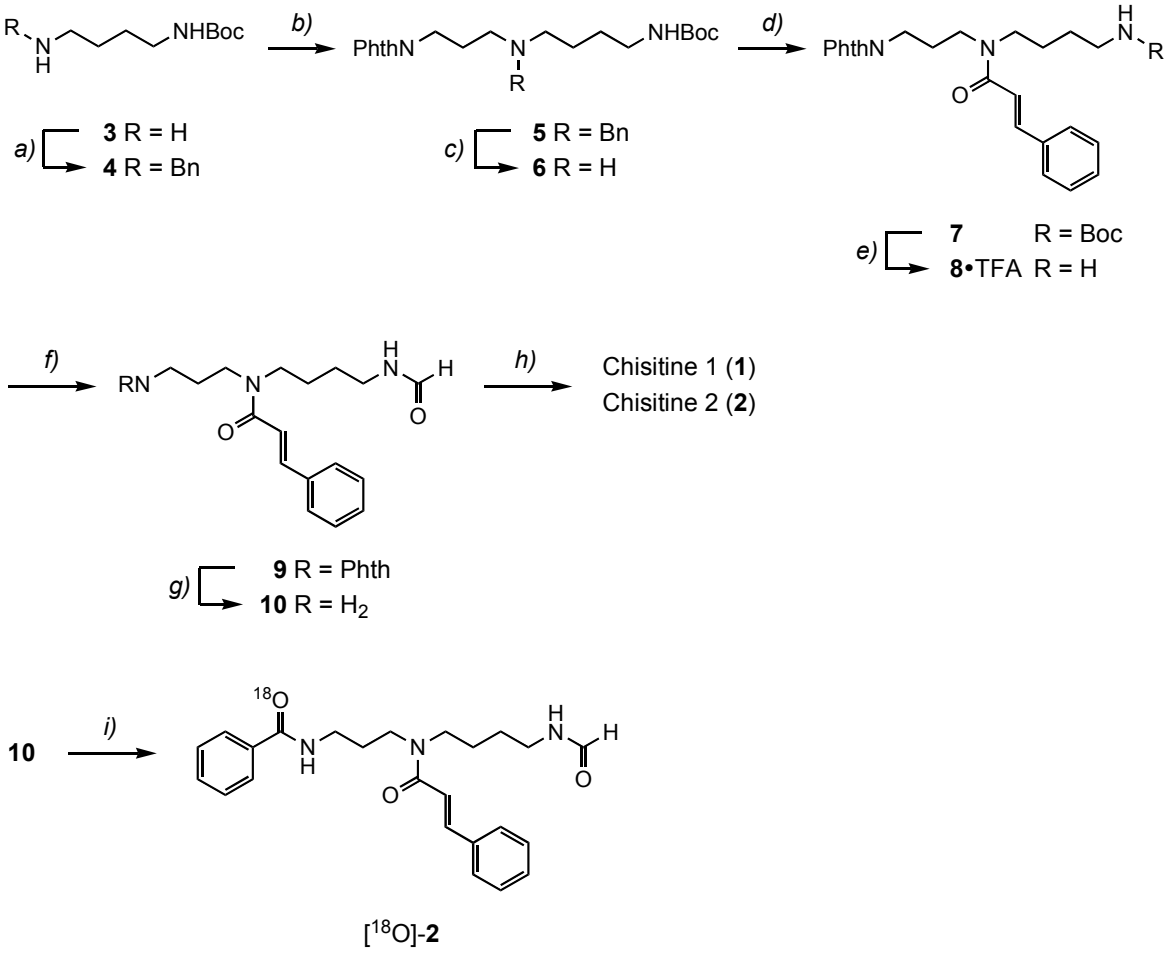
This detailed MS analysis confirmed that the structures of both alkaloids **1** and **2** are correctly assigned. Nevertheless, it seemed important to ensure them by synthesis and also to study their NMR spectra in detail.



Scheme 2. *MS fragmentation of the quasi-molecular ions of chisitine 1 (**1**).*

Synthesis of Chisitines 1 and 2

The two target compounds **1** and **2** were prepared starting from commercially available mono-Boc-protected butane-1,4-diamine **3**, which was benzylated by reductive amination of benzaldehyde to give the intermediate **4** (*Scheme 3*). Alkylation of **4** with *N*-(3-bromopropyl)phthalimide afforded **5**, which was debenzylated to the bis-protected spermidine derivative **6** by hydrogenolysis. Subsequent derivatization of the secondary-amine with cinnamoyl chloride (= (2*E*)-3-phenylprop-2-enoyl chloride) yielded compound **7**.



a) 1. PhCHO, MeOH, r.t., 1 h; 2. $NaBH_4$, 19 h; 95%. *b*) $Br(CH_2)_3NPhth$, K_2CO_3, 70°, 23 h; 78%. *c*) H_2 (4 bar), Pd/C, EtOH, r.t., 19 h; quant. *d*) (*E*)-$C_6H_5CH{=}CHCOCl$, Et_3N, N,N-dimethylaminopyridin-4-amine (DMAP), CH_2Cl_2, r.t., 6 h; 55%. *e*) CF_3COOH, CH_2Cl_2, r.t., 3 h; quant. *f*) $HCO_2(4\text{-}NO_2C_6H_4)$, Et_3N, THF, r.t., 1 h; 82%. *g*) $N_2H_4{\cdot}H_2O$, EtOH, reflux, 2 h; 60%. *h*) MeSCOCl or PhCOCl, pyridine, CH_2Cl_2, r.t., 1 h; 67% (**1**) or 64% (**2**). *i*) 1. $PhC[^{18}O_2]H$, $(COCl)_2$, DMF, CH_2Cl_2, r.t., 2 h; 2. **10**, Et_3N, CH_2Cl_2, r.t., 2 h; 78%.

Scheme 3. *Synthetic route to chisitine 1 (**1**) and 2 (**2**) and labeled analog [^{18}O]-**2**.*

The Boc protecting group was removed by the action of CF_3COOH, and compound **8** was obtained as the CF_3COOH salt. Formylation of **8** by treatment with 4-

nitrophenyl formate gave the spermidine derivative **9**, carrying two of the desired three *N*-substituents. Introduction of the last substituent was achieved after the phthalimide-protecting group was removed by the addition of hydrazine. The resulting primary amine **10** was treated with *S*-methyl carbonochloridothioade to provide the desired alkaloid **1** in 16% overall yield. The reaction of **10** with benzoyl chloride gave compound **2** in 13% overall yield. All analytical data (HPLC-MS, MS/MS, ^{1}H- and ^{13}C-NMR, as well as UV spectra) measured for the two synthetic references were identical with those of the corresponding natural alkaloids **1** and **2**. To study the fragmentation pathway during MS/MS analysis, labeled [^{18}O]-**2** was synthesized analogously by coupling **10** with ^{18}O-labeled benzoyl chloride, which was prepared according to a reported procedure [14].

Conclusion

Two novel spermidine-derived triamide alkaloids were isolated from the leaves of *C. weinlandii*, chisitine 1 (**1**) and chisitine 2 (**2**). The structures of the two compounds were established on the basis of MS and NMR analysis and further confirmed by comparison with their synthetic references. It was shown that **1** and **2** are both present as a 1:1 mixture of two isomers when their NMR spectra are recorded at 300 K. The sites of attachment of the substituents were securely established by the HMBC experiment.

During the mechanistic investigation of the MS fragmentation pathways of the two compounds by ESI-MS/MS, it was observed that acyl-substituted spermidine derivatives undergo dehydration by ring-closure reaction. Another distinction comes from the dependence of the whole dissociation pathways on the lability of the substituents. The replacement of PhCO by a MeSCO moiety, which possesses the good leaving group MeSH, affects the MS/MS fingerprint. The MS/MS fragment ions recorded for the two related compounds arise from alternative pathways. Attempts to elucidate their structure solely on the basis of MS/MS data are, therefore, risky.

We gratefully acknowledge the *Swiss National Science Foundation* for the generous support of this work, Ms. *N. Walch* and the NMR service of the Institute of Organic Chemistry University of Zurich for the measurements. We also acknowledge the *Ministry of Education, Sciences, Sports, and Culture*, and the *Ministry of Health and Welfare, Japan*, for financial help.

Experimental Part

1. *General*. All chemicals were of reagent grade and purchased from *Fluka Chemie AG* or *Merck AG*. All solvents were of analytical grade quality and were used without further purification unless otherwise stated. THF was dried over Na/benzophenone. Hydrogenation: *Parr-Instruments Company Inc.* Flash chromatography (FC): silica gel *Merck 60* (40-63 μm, 230-400 mesh). TLC: *Merck* precoated silica-gel-*60-*F_{254} plates; detection by UV at 254 nm, or by spraying *Schlittler* reagent (H_2PtCl_6/HCl/KI) or ninhydrine reagent and heating at 300° for 1 min. IR Spectra: *Perkin-Elmer 297*; in cm^{-1}. NMR spectra: *Bruker ARX-300* (at 300.13 (^1H) and 75.47 MHz (^{13}C)) at 300 K for all 1D ^1H, ^{13}C, DEPT-135, and DEPT-90 experiments; *DRX-600* (at 600.13 (^1H) and 150.90 MHz (^{13}C)) or *DRX-500* (at 500.13 (^1H) and 125.76 MHz (^{13}C)) at 300 K for the ^1H, ^{13}C,^1H-HSQC, ^{13}C,^1H-HMBC, ^{13}C,^1H-HSQC-TOCSY experiments of **1** and **2**; chemical shifts δ in ppm, referenced against residual non-deuterated solvent; *J* values in Hz. HPLC-UV(DAD)-MS: *Hewlett-Packard 1100* HPLC system with DAD detector (*Hewlett-Packard Co.*, Palo Alto, CA, U.S.A.) equipped with a *HTS-PAL* autosampler (*CTC Analytics*, Zwingen, Switzerland), connected to a *Bruker ESQUIRE-LC* quadrupole ion-trap instrument (*Bruker Daltonik GmbH*, Bremen, Germany) equipped with a combined *Hewlett-Packard* atmospheric-pressure ion (API) source (*Hewlett-Packard Co.*, Palo Alto, CA, U.S.A.). Direct-infusion ESI-MS and ESI-MS^n experiments were performed with a syringe infusion pump (*Cole-Parmer 74900-05*, *Cole-Parmer Instrument Company*, Vernon Hills, IL, U.S.A.).

2. *Isolation of chisitine 1 (**1**) and 2 (**2**).* The leaves of *Chisocheton weinlandii* HARMS were harvested in 1993 in the garden of the Herbarium Bogoriense, Java, Indonesia, and voucher specimens have been deposited at the Herbarium of the Faculty of Pharmaceutical Sciences, Setsunan University.

The crushed leaves (60.0 g) were extracted with MeOH (4 × 1000 ml), and the solvent was distilled. The MeOH extract (10.9 g) was then suspended in H_2O (300 ml) and the aqueous suspension was extracted with AcOEt (4 × 150 ml). After removal of the solvent, the extract (3.7 g) was chromatographed (silica gel, $CHCl_3$/MeOH gradient of increasing MeOH content). Fractions containing **1** and **2** (eluted with $CHCl_3$/MeOH 5:1), were further purified by semi-preparative HPLC to afford pure chisitine 1 (**1**; 26.0 mg) and chisitine 2 (**2**; 15.3 mg). For the analytical data of **1** and **2**,

which are identical in all respects to those of the synthetic references, see *Section 4* below.

3. *HPLC-ESI-MS and ESI-MS Investigation.* Solvents and reagents: MeCN, MeOH (HPLC grade; *Scharlau*, Barcelona, Spain); CF_3COOH *purum* (*Fluka*, Buchs, Switzerland). H_2O was purified with a *Milli-*Q_{RG} apparatus (*Millipore*, Milford, MA, U.S.A). Samples preparation: 0.2 mg of solid material (natural or synthetic) was dissolved in 800 μl of MeOH. For the HPLC-ESI-MS experiments, 10 μl of the stock solutions were injected for the individual runs; for ESI-MS experiments, the solutions were continuously introduced through the electrospray interface at a flow rate of 6 μl min^{-1}. For H/D-exchange experiments, 0.2 mg of solid material was dissolved in 500 μl of a 0.1% solution of DCl in MeOD (*Merck*, Darmstadt, Germany) and stirred at 23° for 15 min prior to use. Column and chromatographic conditions: *Interchrom-Uptisphere-*C_{18}*-HDO* column (*UP3HDO#20QS*, 3 μm, 2.1 × 200 mm; *Interchim*, Montluçon, France); flow rate 0.2 ml min^{-1}; mobile phase: gradient within 30 min from 20 to 50% of solvent *B* and finally within 5 min from 50 to 100% of *B* (solvent *A* = 0.1% solution of CF_3COOH in H_2O, solvent *B* = 0.1% solution of CF_3COOH in MeCN). The UV(DAD) detector was scanning between λ 190 and 400 nm (2 nm step) at a peakwidth of >0.1 min. The MS conditions: nebulizer gas (N_2) 40 psi, dry gas (N_2) 8.5 l min^{-1}, dry temperature 300°, capillary voltage 4200 V, end plate –500 V, capillary exit 86 V, and skimmer 1 18 V; data acquisitions at normal resolution (0.6 Da at half peak height), under ion charge control (ICC) conditions (10 000) in the mass range from *m*/*z* 50 to 600 and an *m*/*z* 39.3 trap drive value. To get representative mass spectra, 8 scans were averaged for each spectrum.

4. *Synthesis of chisitine 1* (**1**) *and 2* (**2**). tert-*Butyl [4-(Benzylamino)butyl]carbamate* (**4**). To a solution of *tert*-butyl (4-aminobutyl)carbamate (**3**; 1.40 g, 7.42 mmol) in MeOH (40 ml), benzaldehyde (0.79 g, 7.43 mmol) was added and the mixture stirred at 23° for 45 min. It was cooled to 0° (ice/water), $NaBH_4$ (0.32 g, 8.46 mmol) was added portionwise over 1 h, and the solution allowed to warm to 23° and stirred for an additional 17 h. After evaporation, the residue was dissolved in H_2O (50 ml) and extracted with CH_2Cl_2 (3 × 50 ml) and the organic phase dried ($MgSO_4$) and evaporated: **4** (1.96 g, 95%). Colorless oil. R_f (CH_2Cl_2/MeOH/25% NH_4OH 9:9:1): 0.64. IR (film): 3340*s*, 2960*s*, 2920*s*, 2850*s*, 2810*s*, 1950*w*, 1875*w*, 1810*w*, 1700*s*, 1610*m*, 1520*s*, 1455*s*, 1390*s*, 1370*s*, 1275*s*, 1250*s*, 1170*s*, 1030*s*, 920*m*, 870*m*, 780*m*, 740*s*, 700*s*. ^{1}H-NMR ($CDCl_3$): 7.31–7.17 (*m*, 5 H); 4.83 (br. *s*, 1 H); 3.73 (*s*, 2 H); 3.08–3.06 (*m*, 2 H); 2.62–2.57 (*m*, 2 H); 1.50–1.46 (*m*, 4 H); 1.39 (*s*, 9 H). ^{13}C-NMR ($CDCl_3$): 156.1 (*s*); 140.6

(*s*); 128.5 (*d*); 128.3 (*d*); 127.1 (*d*); 79.1 (*s*); 54.2 (*t*); 49.1 (*t*); 40.6 (*t*); 28.6 (*q*); 28.0 (*t*); 27.6 (*t*). ESI-MS (MeOH): 279.2 ($[M + H]^+$).

tert-*Butyl {4-{Benzyl[3-(1,3-dihydro-1,3-dioxo-*2H-*isoindol-2-yl)propyl]amino}butyl}carbamate* (**5**). K_2CO_3 (0.52 g, 3.78 mmol) was added to a solution of **4** (0.96 g, 3.43 mmol) in MeCN (15 ml) followed by *N*-(3-bromopropyl)phthalimide (1.01 g, 3.78 mmol), and the heterogeneous mixture was stirred at 70° for 23 h. The solution was poured on H_2O (50 ml) and extracted with CH_2Cl_2 (3 × 50 ml), the combined organic phase dried ($MgSO_4$) and evaporated, and the crude mixture purified by FC (hexane/AcOEt 3:2 → 1:1): pure **5** (1.80 g, 78%). Slightly yellow oil. R_f (CH_2Cl_2/MeOH 1:1): 0.58. IR (film): 3360*s*, 2990*s*, 2900*s*, 2830*s*, 2780*s*, 1940*w*, 1755*s*, 1690*s*, 1600*s*, 1500*s*, 1435*s*, 1380*s*, 1230*s*, 1155*s*, 1020*s*, 900*s*, 870*s*, 855*s*, 705*s*, 685*s*, 655*s*. ^{1}H-NMR ($CDCl_3$): 7.84–7.81 (*m*, 2 H); 7.71–7.68 (*m*, 2 H); 7.31–7.17 (*m*, 5 H); 4.74 (br. *s*, 1 H); 3.69 (*t*, *J* = 7.6, 2 H); 3.52 (*s*, 2 H); 3.07–3.06 (*m*, 2 H); 2.50–2.40 (*m*, 4 H); 1.83 (*quint.*, *J* = 7.3, 2 H); 1.50–1.45 (*m*, 4 H); 1.43 (*s*, 9 H). ^{13}C-NMR ($CDCl_3$): 168.5 (*s*); 156.2 (*s*); 139.8 (*s*); 134.0 (*d*); 132.4 (*s*); 129.1 (*d*); 128.3 (*d*); 127.0 (*d*); 123.3 (*d*); 79.1 (*s*); 58.8 (*t*); 53.6 (*t*); 51.4 (*t*); 40.7 (*t*); 36.6 (*t*); 28.6 (*q*); 28.0 (*t*); 26.3 (*t*); 24.7 (*t*). ESI-MS (MeOH + NaI): 488.3 ($[M + Na]^+$).

tert-*Butyl {4-{[3-(1,3-Dihydro-1,3-dioxo-*2H-*isoindol-2-yl)propyl]amino}butyl}carbamate* (**6**). A mixture of **5** (1.18 g, 2.54 mmol) and Pd/C (200 mg) in EtOH (100 ml) was stirred for 19 h at 23° under a H_2 pressure of 4 bar [15]. The catalyst was filtered off *via Celite*® and washed with MeOH (150 ml). The combined filtrate was evaporated: **6** (1.26 g, quant.). Colorless solid. R_f (CH_2Cl_2/MeOH 1:1): 0.61. IR (film): 3370*m*, 2940*m*, 2790*m*, 2440*w*, 2780*s*, 1770*w*, 1705*s*, 1690*s*, 1600*w*, 1530*m*, 1465*m*, 1440*m*, 1400*m*, 1365*m*, 1270*m*, 1250*m*, 1175*m*, 715*m*. ^{1}H-NMR ($CDCl_3$): 7.84–7.81 (*m*, 2 H); 7.72–7.69 (*m*, 2 H); 5.14 (br. *s*, 1 H); 3.85 (*t*, *J* = 6.5, 2 H); 3.17–3.11 (*m*, 2 H); 3.04–2.95 (*m*, 4 H); 2.32–2.27 (*m*, 2 H); 1.95–1.85 (*m*, 2 H); 1.66–1.47 (*m*, 2 H); 1.41 (*s*, 9 H). ^{13}C-NMR ($CDCl_3$): 168.6 (*s*); 156.5 (*s*); 134.3 (*d*); 132.1 (*s*); 123.6 (*d*); 79.5 (*s*); 47.8 (*t*); 45.8 (*t*); 39.9 (*t*); 35.2 (*t*); 28.6 (*q*); 27.3 (*t*); 25.8 (*t*); 23.3 (*t*). ESI-MS (MeOH): 320.2 (5), 376.3 (100, $[M + H]^+$), 398.2 (6, $[M + Na]^+$).

tert-*Butyl {4-{[3-(1,3-Dihydro-1,3-dioxo-*2H-*isoindol-2-yl)propyl][(*2E*)-3-phenylprop-2-enoyl]amino}butyl}carbamate* (**7**). According to [16], Et_3N (3.67 g, 36.29 mmol) and DMAP (42 mg, 0.34 mmol) were added to a solution of **6** (948 mg, 2.54 mmol) in CH_2Cl_2 (25 ml). After stirring at 23° for 30 min, (2*E*)-3-phenylprop-2-enoyl chloride (847 mg, 5.08 mmol) was added, and the mixture was stirred at 23° for an additional

6 h. The solution was washed with saturated aqueous NH_4Cl solution (3 × 30 ml) followed by saturated aqueous Na_2CO_3 solution (3 × 30 ml). The organic layer was dried ($MgSO_4$) and evaporated. The crude mixture was purified by FC (hexane/AcOEt 1:1 → 2:3): pure **7** (707 mg, 55%). Yellow oil. R_f (hexane/AcOEt 1:1): 0.11. IR (film): 3350*m*, 3070*m*, 2980*s*, 2940*s*, 2870*m*, 1775*s*, 1710*s*, 1655*s*, 1610*s*, 1525*s*, 1475*s*, 1460*s*, 1445*s*, 1405*s*, 1375*s*, 1340*s*, 1280*s*, 1260*s*, 1180*s*, 1150*m*, 1100*m*, 1080*m*, 1045*m*, 985*m*, 900*m*, 865*m*, 775*s*, 730*s*, 695*m*, 675*m*. ^{1}H-NMR ($CDCl_3$): 7.88–7.82 (*m*, 2 H); 7.74–7.63 (*m*, 3 H); 7.52–7.43 (2 *m*, 2 H); 7.36–7.31 (*m*, 3 H); 6.83, 6.76 (2 *d*, *J* = 15.4 each, ratio 1:1, 1 H); 4.65 (br. *s*, 1 H); 3.77–3.72 (*m*, 2 H); 3.55–3.44 (*m*, 4 H); 3.18–3.12 (*m*, 2 H); 2.10–1.95 (*m*, 2 H); 1.75–1.62 (*m*, 2 H); 1.57–1.47 (*m*, 2 H); 1.41 (*s*, 9 H). ^{13}C-NMR ($CDCl_3$): 168.4 (*s*); 166.5, 166.4 (2 *s*); 156.2 (*s*); 143.0 (*d*); 135.5 (*s*); 134.3, 134.1 (2 *d*); 132.3, 132.1 (2 *s*); 129.7, 128.9, 128.0 (3 *d*); 123.6, 123.4 (2 *d*); 117.5, 117.3 (2 *d*); 79.4 (*s*); 48.0, 46.6, 46.1, 44.6 (4 *t*); 40.2 (*t*); 36.2, 35.7 (2 *t*); 29.0 (*t*); 28.6 (*q*); 27.8, 27.6, 27.4, 27.2, 25.4 (5 *t*). ESI-MS (MeOH + NaI): 528.3 ($[M + Na]^+$).

*4-{[3-(1,3-Dihydro-1,3-dioxo-*2H*-isoindol-2-yl)propyl][(*2E*)-3-phenylprop-2-enoyl]amino}-butan-1-ammonium Trifluoroacetate* (**8**). CF_3COOH (1.58 g, 13.85 mmol) was added dropwise to a solution of **7** (700 mg, 1.39 mmol) in CH_2Cl_2 (10 ml), and the mixture was stirred at 23° for 3 h. Evaporation gave **8** (929 mg, quant.). Brownish oil. R_f (CH_2Cl_2/MeOH 1:1): 0.50. IR (film): 3310*m*, 1805*s*, 1745*s*, 1680*s*, 1615*m*, 1535*m*, 1505*m*, 1475*m*, 1440*s*, 1420*m*, 1370*m*, 1340*m*, 1240*s*, 1205*s*, 1110*m*, 1065*m*, 1010*m*, 850*m*, 830*m*, 805*m*, 760*s*, 745*m*. ^{1}H-NMR ($CDCl_3$): 7.74–7.62 (*m*, 4 H); 7.47 (*d*, *J* = 15.4, 1 H); 7.35–7.18 (*m*, 5 H); 6.64 (*d*, *J* = 15.4, 1 H); 3.68–3.58 (*m*, 2 H); 3.47–3.41 (*m*, 4 H); 3.12–3.10 (*m*, 2 H); 2.03–1.94, 1.92–1.87 (2 *m*, ratio 3:1, 2 H); 1.72–1.65 (*m*, 4 H). ^{13}C-NMR ($CDCl_3$): 169.5, 169.3 (2 *s*); 168.9 (*s*); 160.7 (*q*, *J*(CO,F) = 41.2); 146.0 (*d*); 134.8, 134.7 (2 *d*); 134.2 (*s*); 131.7 (2 *s*); 130.9, 129.2, 128.3 (3 *d*); 123.8, 123.7 (2 *d*; 115.2 (*q*, *J*(C,F) = 286.9); 115.3, 115.2 (2 *d*); 48.6, 47.0, 46.5, 45.8 (4 *t*); 40.5, 40.2 (2 *t*); 35.9, 35.5 (2 *t*); 28.3, 27.4, 26.4, 24.7, 24.4, 24.0 (6 *t*). ESI-MS (MeOH): 406.3 (100, $[M + H]^+$), 428.2 (6, $[M + Na]^+$).

*(*2E*)-*N*-[3-(1,3-Dihydro-1,3-dioxo-*2H*-isoindol-2-yl)propyl]-*N*-[4-(formylamino)butyl]-3-phenylprop-2-enamide* (**9**). According to [17], 4-nitrophenyl formate (231 mg, 1.38 mmol) in THF (4 ml) was added dropwise to a solution of **8** (700 mg, 1.35 mmol) and Et_3N (684 mg, 6.76 mmol) in THF (10 ml). The mixture was stirred at 23° for 1 h. Evaporation and purification of the crude product by FC (CH_2Cl_2/MeOH 93:7 → 9:1) gave pure **9** (481 mg, 82%). Yellowish oil. R_f (CH_2Cl_2/MeOH 9:1): 0.39. IR (film): 3370*m*, 3140*m*, 3020*m*, 2950*m*, 1810*s*, 1750*s*, 1715*s*, 1690*s*, 1640*s*, 1575*m*, 1540*m*, 1475*s*, 1440*s*, 1420*s*, 1370*m*, 1345*m*, 1230*m*, 1175*m*, 1115*m*, 1070*m*, 1020*m*, 930*m*, 805*s*, 760*s*. ^{1}H-

NMR ($CDCl_3$): 8.16 (br. *s*, 1 H); 7.86–7.81 (*m*, 2 H); 7.74–7.62 (*m*, 3 H); 7.51–7.31 (*m*, 5 H); 6.81, 6.76 (2 *d*, *J* = 15.4 each, ratio 1:1, 1 H); 6.40, 6.01 (2 br. *s*, 1 H); 3.78–3.71 (*m*, 2 H); 3.55–3.43 (*m*, 4 H); 3.38–3.23 (*m*, 2 H); 2.10–1.92 (*m*, 2 H); 1.68–1.52 (*m*, 4 H). ^{13}C-NMR ($CDCl_3$): 168.4 (*s*); 166.6 (*s*); 164.7, 161.5 (2 *d*); 143.3, 143.1 (2 *d*); 135.4, 135.3 (2 *s*); 134.4, 134.3 (2 *d*); 132.2, 132.1 (2 *s*); 129.8, 129.0, 128.0, 123.6, 123.4 (5 *d*); 117.4, 117.1 (2 *d*); 48.1, 46.4, 46.3, 44.8 (4 *t*); 37.7 (*t*); 36.2, 35.7 (2 *t*); 29.0, 27.3, 27.2, 26.3, 25.5 (5 *t*). ESI-MS (MeOH + NaI): 456.3 ($[M + Na]^+$).

*(2*E*)-*N*-(3-Aminopropyl)-*N*-[4-(formylamino)butyl]-3-phenylprop-2-enamide* (**10**). According to [16], $N_2H_4 \cdot H_2O$ (412 mg, 8.23 mmol) was added to a solution of **9** (360 mg, 0.83 mmol) in EtOH (10 ml) and the mixture was stirred under reflux for 2 h. After cooling, the solvent was evaporated, the residue treated with 2N aqueous HCl solution (10 ml), the mixture filtered, and the filtrate basified with saturated aqueous Na_2CO_3 solution (15 ml). The aqueous layer was extracted with CH_2Cl_2 (3 × 20 ml) and the combined organic layers were dried ($MgSO_4$) and evaporated: **10** (152 mg, 60%). Yellowish oil. R_f (CH_2Cl_2/MeOH 1:1): 0.05. IR (film): 3360*m*, 3130*m*, 3010*m*, 2940*m*, 1710*s*, 1685*s*, 1635*s*, 1535*m*, 1495*s*, 1465*s*, 1420*m*, 1365*m*, 1340*m*, 1320*m*, 1270*m*, 1240*m*, 1220*m*, 1020*m*, 805*m*, 750*m*, 725*m*. ^{1}H-NMR ($CDCl_3$): 8.14 (*s*, 1 H); 7.67–7.66, 7.62–7.60, 7.57–7.56 (3 *m*, ratio 43:50:7, 1 H); 7.51–7.42, 7.34–7.28 (2 *m*, 5 H); 6.96, 6.79, 6.41 (3 *d*, *J* ≈ 15.4 each, 1 H); 6.77, 6.54 (2 br. *s*, 1 H); 3.51–3.39 (*m*, 4 H); 3.34–3.21 (*m*, 2 H); 2.76–2.58 (*m*, 2 H); 1.74–1.50 (*m*, 8 H). ^{13}C-NMR ($CDCl_3$): 166.8, 166.7 (2 *s*); 164.8, 161.6 (2 *d*); 142.9, 142.7 (2 *d*); 135.4, 135.3 (2 *s*); 129.7, 128.9, 128.0 (3 *d*); 117.9, 117.4 (2 *d*); 47.6, 46.2, 45.8, 43.9, 39.3, 39.1, 37.7, 37.5 (8 *t*); 33.2, 31.4 (2 *t*); 27.1, 26.9, 26.4, 25.4 (4 *t*). ESI-MS (MeOH + HCOOH): 286.2 (6), 304.2 (100, $[M + H]^+$), 326.2 (6, $[M + Na]^+$).

*S-Methyl{3-{[4-(Formylamino)butyl][(2*E*)-3-phenylprop-2-enoyl]amino}propyl}carbamothioate* (= *chisitine 1*; **1**). In analogy to [18], *S*-methyl carbonochloridothioate (43 mg, 0.39 mmol) was added dropwise to a solution of **10** (107 mg, 0.35 mmol) and pyridine (31 mg, 0.39 mmol) in CH_2Cl_2 (3 ml) and the mixture was stirred at 23° for 1 h. Evaporation and purification of the crude mixture by FC (CH_2Cl_2/MeOH 95:5 → 9:1) gave pure **1** (90 mg, 67%). Yellowish oil that solidified upon standing at 4°. M.p. 89–90°. R_f (CH_2Cl_2/MeOH 9:1): 0.31. IR (film): 3350*s*, 3120*m*, 3000*m*, 2940*m*, 1705*s*, 1685*s*, 1635*s*, 1570*s*, 1535*s*, 1520*s*, 1495*s*, 1470*s*, 1420*s*, 1365*m*, 1340*m*, 1255*s*, 1160*m*, 1015*m*, 800*m*, 745*m*, 725*m*. NMR: *Tables 1* and *2*. ESI-MS (MeOH): 378.2 (26, $[M + H]^+$), 400.3 (88, $[M + Na]^+$), 416.1 (100, $[M + K]^+$). MS^n: *Table 3*. HPLC-UV-MS: t_R = 23.8 min; λ_{max} 204, 218, 282 nm.

N-*{3-{[4-(Formylamino)butyl][(*2E*)-3-phenylprop-2-enoyl]amino}propyl}benzamide* (= *chisitine 2*; **2**). According to [19], benzoyl chloride (18 mg, 0.13 mmol) was added dropwise to a solution of **10** (36 mg, 0.12 mmol) and pyridine (10 mg, 0.13 mmol) in CH_2Cl_2 (2 ml) and the mixture was stirred at 23° for 2 h. Evaporation and purification of the crude mixture by FC (CH_2Cl_2/MeOH 95:5 → 9:1) gave pure **2** (31 mg, 64%). Yellow oil that solidified upon standing at 4°. M.p. 102–103°. R_f (CH_2Cl_2/MeOH 9:1): 0.25. IR (film): 3290*s*, 3050*m*, 2920*s*, 2860*m*, 2230*w*, 1645*s*, 1595*s*, 1535*s*, 1485*s*, 1455*s*, 1430*s*, 1380*s*, 1325*s*, 1300*s*, 1240*m*, 1190*s*, 1120*m*, 1090*m*, 1070*m*, 1025*m*, 975*m*, 910*m*, 855*m*, 800*m*, 765*s*, 700*s*. NMR: *Tables 1* and *2*. ESI-MS (MeOH): 408.3 (63, $[M + H]^+$), 430.2 (100, $[M + Na]^+$), 446.1 (70, $[M + K]^+$). MS^n: *Table 3*. HPLC-UV-MS: t_R = 25.0 min; λ_{max} 220, 280 nm.

N-*{3-{[4-(Formylamino)butyl][(*2E*)-3-phenylprop-2-enoyl]amino}propyl}benz[^{18}O]amide* (*[^{18}O]*-**2**). $(COCl)_2$ (9 mg, 0.07 mmol) was added to a cold (ice/water) solution of [$^{18}O_2$]benzoic acid (8 mg, 0.07 mmol; prepared according to [14]) and DMF (*ca.* 0.5 ml, catalyst) in CH_2Cl_2 (1.5 ml), and the resultant mixture allowed to warm to 23° and stirred for an additional 2 h. After evaporation of the oil, the residue was dissolved in CH_2Cl_2 (1 ml) and the mixture cooled to 0° (ice/water) and treated with a solution of **10** (10 mg, 0.03 mmol) and Et_3N (7 mg, 0.07 mmol) in CH_2Cl_2 (1.5 ml). After stirring at 23° for 2 h, the solvent was evaporated and the crude mixture was purified by preparative TLC (CH_2Cl_2/MeOH 9:1): [^{18}O]-**2** (11 mg, 78%). R_f (CH_2Cl_2/MeOH 9:1): 0.25. ESI-MS (MeOH + NaI): 432.3 ($[M + Na]^+$). MS^n: *Table 3*.

References

[1] S. Bienz, R. Detterbeck, C. Ensch, A. Guggisberg, U. Häusermann, C. Meisterhans, B. Wendt, C. Werner, M. Hesse, *Alkaloids* **2002**, *58*, 83.

[2] J. D. Connolly, C. Labbé, D. S. Rycroft, D. A. H. Taylor, *J. Chem. Soc., Perkin Trans. 1* **1979**, 2959.

[3] M. Bordoloi, B. Saikia, R. K. Mathur, B. N. Goswami, *Phytochemistry* **1993**, *34*, 583.

[4] R. D. Yadav, J. C. S. Kataky, R. K. Mathur, *Indian J. Chem.* **1999**, *38B*, 1359.

[5] P. J. Gunning, L. B. Jeffs, M. B. Isman, G. H. N. Towers, *Phytochemistry* **1994**, *36*, 1245.

[6] K. Drandarov, M. Hesse, *Tetrahedron Lett.* **2002**, *43*, 5025.

[7] H. Greger, G. Zechner, O. Hofer, S. Vajrodaya, *J. Nat. Prod.* **1996**, *59*, 1163.

[8] N. M. Cuong, W. C. Taylor, T. V. Sung, *Phytochemistry* **1999**, *52*, 1711.

[9] E. Saifah, J. Puripattanavong, K. Likhitwitayawuid, G. A. Cordell, H. Chai, J. M. Pezzuto, *J. Nat. Prod.* **1993**, *56*, 473.

[10] H. Bosshardt, M. Hesse, *Angew. Chem.* **1974**, *86*, 256; *Angew. Chem. Int. Ed. Engl.* **1974**, *13*, 252.

[11] A. Guggisberg, M. Hesse, *Alkaloids* **1983**, *22*, 85.

[12] L. Bigler, M. Hesse, *J. Am. Soc. Mass Spectrom.* **1995**, *6*, 634.

[13] K. D. Ballard, S. J. Gaskell, *J. Am. Soc. Mass Spectrom.* **1993**, *4*, 477.

[14] S. F. Wnuk, S. M. Chowdhury, P. I. Garcia, Jr., M. J. Robins, *J. Org. Chem.* **2002**, *67*, 1816.

[15] I. R. Marsh, M. Bradley, *Tetrahedron* **1997**, *53*, 17317.

[16] C. Jentgens, R. Hofmann, A. Guggisberg, S. Bienz, M. Hesse, *Helv. Chim. Acta* **1997**, *80*, 966.

[17] L. R. Orelli, M. B. Garcia, F. Niemevz, I. A. Perillo, *Synth. Commun.* **1999**, *29*, 1819.

[18] S. Hinterberger, O. Hofer, H. Greger, *Tetrahedron* **1998**, *54*, 487.

[19] R. Detterbeck, M. Hesse, *Tetrahedron* **2002**, *58*, 6887.

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List of Publications

Nikolay Manov, Manuel Tzouros, Sergiy Chesnov, Laurent Bigler, and Stefan Bienz. Solid-Phase Synthesis of Polyamine Spider Toxins and Correlation with the Natural Products by HPLC-MS/MS. *Helv. Chim. Acta* **2002**, *85*, 2827-2846.

Nikolay Manov, Manuel Tzouros, Laurent Bigler, and Stefan Bienz. Solid-Phase Synthesis of ^{15}N-Labeled Acylpentamines as Reference Compounds for the MS/MS Investigation of Spider Toxins. *Tetrahedron* **2004**, *60*, 2387-2391.

Manuel Tzouros, Laurent Bigler, Stefan Bienz, Manfred Hesse, Akira Inada, Hiroko Murata, Yuka Inatomi, Tsutomu Nakanishi, and Dedy Darnaedi. Two New Spermidine Alkaloids from *Chisocheton weinlandii*. *Helv. Chim. Acta* **2004**, *87*, 1411-1425.

Manuel Tzouros, Nikolay Manov, Stefan Bienz, and Laurent Bigler. Tandem Mass Spectrometric Investigation of Acylpolyamines of Spider Venoms and their ^{15}N-Labeled Derivatives. *J. Am. Soc. Mass Spectrom.* **2004**, *15*, 1636-1643.

Manuel Tzouros, Sergiy Chesnov, Stefan Bienz, Manfred Hesse, and Laurent Bigler. New Linear Polyamine Derivatives in Spider Venoms. *Toxicon* **2005**, *46*, 0000.

Manuel Tzouros, Sergiy Chesnov, Stefan Bienz, Manfred Hesse, and Laurent Bigler. Characterization of Linear Polyamines in Spider Venoms by Liquid Chromatography/Atmospheric-Pressure Chemical Ionization Tandem-Mass Spectrometry. In prep.

List of Scientific Presentations

Manuel Tzouros, Nikolay Manov, Sergiy Chesnov, Laurent Bigler, Stefan Bienz, and Manfred Hesse. Is HPLC-Tandem Mass Spectrometry a Suitable Method for the Characterization of New Acylpolyamine Derivatives? Swiss Group for Mass Spectrometry, Rigi Meeting **2002**, Beatenberg, oral presentation.

Manuel Tzouros, Nikolay Manov, Sergiy Chesnov, Laurent Bigler, Stefan Bienz, and Manfred Hesse. Characterization of New Acylpolyamine Derivatives by Tandem-Mass Spectrometry. 36. Diskussionstagung der Deutsche Gesellschaft für Massenspektrometrie **2003**, Münster (D), poster.

Manuel Tzouros, Stefan Bienz, and Laurent Bigler. Tandem-Mass Spectrometry as a Key Tool for the Structure Elucidation of Spermidine Alkaloids. Swiss Chemical Society, Fall Meeting **2003**, Lausanne, oral presentation.